Part B

Hazard Assessment and Review of Available Studies

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Chapter 4

4 Human Epidemiological Studies

Several epidemiologic studies have examined cancers among populations with exposures relevant to the assessment of atrazine, especially among farmers or farm residents (Table 4-1). Most are case control studies, although others include ecologic investigations and a worker mortality study associated with triazine manufacturing.

4.1 Colon Cancer

Associations between herbicide use by farmers and colon cancer in Kansas was investigated in a case-control study (Hoar *et al.*, 1985). Starting with 57 cases of colon cancer and 948 controls, the odds ratio (OR) for the subset using triazine herbicides was 1.4 (95% C.I. 0.2-7.9). The sample size in this group was very small with only two cancer cases with confirmed exposure to triazines and 43 controls. The study author stated that the data did not support an association between colon cancer and herbicide exposure

An ecologic study of ecodistricts in Canada compared triazine exposures and cancer incidences (Van Leeuwen *et al.*, 1999). Association of triazine exposure with several cancers, including colon, were examined. Significant negative associations were found in both sexes ($p = 0.041$ in females and 0.006 in males).

4.2 Non-Hodgkins Lymphoma

Zahm *et al.* (1993b) pooled results of three case-referent studies conducted in three midwestern states that investigated atrazine exposure in the development of non-Hodgkins lymphoma (NHL). Starting with 993 males with NHL and 2918 controls, persons were queried as to their pesticide exposures. An OR = 1.4 (95% CI 1.1-1.8) was found for atrazine use and NHL. However, when adjustments were made for use of 2,4-dichloroacetic acid and organophosphate use, the OR = 1.2 (0.9-1.7). The authors concluded that there was essentially no risk of NHL attributable to farm use of atrazine.

NHL was investigated among women who lived or worked on a farm in eastern Nebraska (Zahm *et al.*, 1993a). The OR for those who reported that they lived on a farm where atrazine was used was 1.4 (95% CI 0.6 - 3.0) with 11 cases and 31 controls. For those women who reported having personally used atrazine the OR = 2.2 (0.1 - 31.5) with only one case and two controls. Cyanazine was also investigated and the OR for NHL and those who reported using cyanazine was 1.3 (0.3 - 4.5) with four cases and 12 controls. The study author noted that there were too few subjects in any of these analyses to adequately assess associations.

Correlations were made between pesticide use (1993) and NHL incidence (1988-1992) among California counties (Mills, 1998). There were negative correlations for white males and females and Hispanic males; the correlation was positive for Hispanic females (0.12), but it was not statistically-significant.

An ecologic study of ecodistricts in Canada compared triazine exposures and cancer incidences (Van Leeuwen *et al.*, 1999). Association of triazine exposure with several cancers, including NHL, were examined. No association was found in females and a negative association was found in males.

A mortality study of workers in two triazine manufacturing plants that was supplied to EPA (Delzell and Sathiakumar, 1996) did not find any significant excesses of deaths for any disease category. There were, however, two cases of NHL in plant workers - one of whom was relatively young (31 years). These two cases do not provide evidence of an association between atrazine exposure and NHL, but do indicate that further follow-up of workers in these triazine manufacturing plants would be helpful.

4.3 Soft Tissue Sarcoma

A population-based case-control study of soft-tissue sarcoma (STS) in Kansas demonstrated that there was no increased risk among farmers (Hoar *et al*., 1986). The lack of association persisted when years of herbicide use or frequency of herbicide use were considered. Analyses examining atrazine specifically were not conducted.

A study previously described above (Mills, 1998) examined associations between pesticide use and STS. Positive correlations were not noted for either males or females for atrazine use and STS.

4.4 Other Hematologic Cancers: Hodgkins Disease, Leukemia, Multiple Myeloma

Hoar *et al*. (1986) examined associations between herbicide use and Hodgkins disease (HD). An OR for atrazine exposure and HD was not reported in the study, but for herbicide use in general the OR was 0.9 (95% CI 0.5 - 1.5). The study author did not consider herbicide exposure to be associated with HD.

Association of pesticide exposure (including atrazine and cyanazine) to leukemia was investigated in a population-based case-control study of adult white men in Iowa and Minnesota (Brown *et al.*, 1990). The OR in those who reported mixing, loading and applying atrazine or cyanazine was 1.0 (0.6 - 1.5) for atrazine and 0.9 (0.5 - 1.6) for cyanazine.

Mills (1998) also examined associations between pesticide use and leukemia. Positive correlations were not noted for either Hispanic or white males or females for atrazine use and leukemia.

Triazine exposure and multiple myeloma in Iowa farmers was investigated in a case-control study by Burnmeister (1990). The OR for triazine use and multiple myeloma was 1.29 and was not significant.

4.5 Ovarian Cancer

A case-control study of epithelial ovarian cancer was conducted in woman between the ages of 20 and 69 who lived in a province in Italy where triazine herbicides are used in farming (Donna *et al.*, 1989). A relative risk (RR) of 2.7 (95% CI 1.0 - 6.9) was found for subjects who reported that they definitely had been exposed to triazine herbicides; the sample size in this subgroup was seven cases and seven controls. The authors considered that there was some risk of ovarian cancer among women who were exposed to triazines.

An ecologic study of ecodistricts in Canada compared triazine exposures and cancer incidences (Van Leeuwen *et al.*, 1999). Association of triazine exposure with several cancers, including ovarian, were examined. No association between atrazine exposure and ovarian cancer was found.

4.6 Breast Cancer

An ecologic study of counties in Kentucky compared measures of triazine exposures (ground and surface water measurements, and acres of land planted with corn and triazine application rates) with state cancer incidences (Kettles *et al.*, 1997). For the years 1993-1994, the OR = 1.14 (95% CI 1.08 - 1.19) for counties with medium triazine exposure, compared with OR = 1.2 (1.13 - 1.28) for high exposure counties. Although only slightly greater than 1.0, these OR's were still statistically-significant ($p<0.0001$).

4.7 Prostate Cancer

Pounds of atrazine applied in California counties during the calendar year 1993 were compared with state cancer incidences (Mills, 1998). A statistically-significant correlation coefficient of 0.67 was obtained for blacks and prostate cancer. The correlation coefficients for whites, Asians and Hispanics were not statistically significant.

4.8 Stomach Cancer

An ecologic study of ecodistricts in Canada compared triazine exposures and cancer incidences (Van Leeuwen *et al.*, 1999). Association of triazine exposure with several cancers, including stomach, were examined. A significant positive association was found in both sexes ($p = 0.242$ in females and 0.046 in males).

4.9 Summary

Colon cancer does not appear to be associated with triazine exposure as suggested by a non-significant OR of 1.4 in a single study (Hoar, 1985). The sample size in this study for triazine use was, however, very small. An ecologic study found negative associations between atrazine exposure and colon cancer in both sexes (Van Leeuwen *et al.*, 1999).

Soft tissue sarcoma does not appear to be associated with atrazine exposure. The correlation coefficient from Mills, 1998 was not significant and the OR from Hoar *et al.* (1986) was 0.9. Hodgkins disease does not appear to associated with atrazine as indicated by an OR of 0.9 in Hoar *et al.* (1986).

Two studies examined an association of atrazine to leukemia. One found an OR of 1.0 (Brown, *et al.*, 1990) and the other found a correlation coefficient which was not significant (Mills, 1998). Leukemia does not appear to be associated with atrazine exposure.

Triazine exposure and multiple myeloma do not appear to be associated. The OR in a single study was 1.29 (Burnmeister, 1990). This OR was not significant.

The results in regards to non-Hodgkins lymphoma (NHL) are mixed, but overall indicative of a lack of association of triazines with NHL. One study found a significant association with an OR of 2.5 (Hoar, *et al.*, 1985). When the data from this study is pooled with data from two other studies, a much lower OR of 1.4 is found (Zahm, 1993b). The Zahm, 1993b study represents the pooled data from three separate studies. Thus, the sample size is quite large -- 130 cases and 249 controls. The positive Hoar *et al*., 1985 study, by comparison, had 14 cases and 43 controls. An OR of 2.2 was found for women who had reported using atrazine (Zahm, 1993a). The sample size in this study was very small with only one case and two controls. A fourth study failed to find any positive correlations for either Hispanics or white males or females for atrazine use and NHL (Mills, 1998). The low OR in the pooled study with a large sample size, combined with the lack of positive correlations in Mills, 1998, indicates that atrazine has not yet been clearly shown to be associated with atrazine. Further research in this area is desirable though given the positive association seen in Hoar, *et al*., 1985 and the previously described incidence of two cases of NHL in workers employed at triazine manufacturing plants.

The most clear associations between atrazine and cancer occurs for ovary, breast and prostate cancer. Interestingly, all three of these cancers are known to be hormone-responsive. These associations should not be considered as conclusive evidence of an association of atrazine with these tumor types though.

Two of the associations (breast and prostate cancer) were found in ecologic studies. Ecologic studies contain inherent limitations and causal effects can not be found from ecologic studies. The primary limitation of an ecologic study is that chemical exposure can not be confirmed. Exposure in epidemiologic studies can sometimes be uncertain. For example, interview-based studies rely on a persons memory to determine exposure and a recall bias may be evident. But in ecologic studies exposure is most uncertain. In ecologic studies the researcher has no idea at all if the persons who contracted cancer had any exposure at all to the chemical in question. The researcher only knows that the person lived in a county in which the chemical was used or lived in a county which had chemical contamination of the water supplies.

The association with ovarian cancer seen in Donna, *et al*., 1989, is also weakened by confounding variables. The most dramatic weakness in this study is the small sample size in the "defiantly exposed" group. This group consists of only seven cases and seven controls. Furthermore, close examination of this group reveals that it may be even smaller. A description of the exposure of the seven women in the "defiantly exposed" group is included as an appendix to the study. Examination of the descriptions in the appendix show that three out of the seven did not actually recall exposures to triazines at all. Rather, these three noted only that they had worked in fields where herbicides were used, but that they could not recall the names of the herbicides. The small sample size limits of the defiantly exposed group weakens the conclusions from this study.

4.10 Conclusions

The results of the human epidemiology studies do not provide clear evidence of an association between triazines and cancer. Some of the studies, particularly those in which hormone-responsive cancers such as breast, ovary and prostate, were examined, are suggestive of a possible association. There is also suggestive evidence of a possible association of triazine exposure and NHL. Further epidemiologic research is needed - especially in the area of hormone-responsive cancers.

Table 4-1. Odds Ratios (OR), Risk Ratio (RR) or Correlation Coefficient

Study	Cancer	Risk Measure or Correlation
Hoar *et al.*, 1985	Colon	*Triazines*: OR = 1.4 95% CI= 0.2 - 7.9
Hoar *et al.*, 1986	Non-Hodgkins lymphoma[1]	*Triazines*: OR = 2.5 95% CI= 1.2 - 5.4
Donna *et al.*, 1989	Ovary	*Triazines*: RR = 2.7 95% CI= 1.0 - 6.9
Brown *et al.*, 1990	Leukemia	*Atrazine*: OR = 1.0 95% CI= 0.6 - 1.5 *Cyanazine*: OR = 0.9 95% CI= 0.5 - 1.6
Burmeister, 1990	Multiple myeloma	*Triazines*: OR = 1.3 95% CI not given
Zahm *et al.*, 1993a	Non-Hodgkins lymphoma in women	*Use on farm*: OR = 1.4 95% CI= 0.6 - 3.0 *Personal use*: OR = 2.2 95% CI= 0.1 - 31.5
Zahm *et al.*, 1993b	Non-Hodgkins lymphoma in men	*Atrazine*: OR = 1.4 95% CI= 1.1 - 1.8
Kettles *et al.*, 1997	Breast	*Triazine exposure* *Medium:* OR = 1.14 95% CI = 1.08 - 1.19 *High*: OR = 1.2 95% CI = 1.13-1.28
Mills, 1998	Prostate[2]	Statistically-significant correlation = 0.67 between atrazine exposure and prostate cancer in blacks, but not in whites, Hispanics and Asians
Van Leuwen *et al*., 1999	Stomach[3]	Female p= +0.242 Male p = +0.046

[1]Soft tissue sarcoma and Hodgkins disease were also investigated and determined, by the study author, not to have a significant association with atrazine exposure.

[2]Leukemia, non-Hodgkins lymphoma, soft tissue, brain and testis cancer were examined, but a significant correlation was seen only with prostate cancer.

[3] Bladder, colon, brain, NHL and ovary cancer were also examined, but either no association or a negative association was seen in each case.

Chapter 5

5 Chronic Rodent Bioassay Studies

The carcinogenicity of atrazine in the female Sprague-Dawley (SD) rat has been confirmed in several two-year bioassays. These studies show that atrazine exposure results in an increased incidence and an early onset of mammary tumors in female SD rats (Mayhew *et al.*, 1986; Thakur, 1991a[1]; Thakur, 1992a; Morseth, 1998; Pettersen and Turnier, 1995). No tumor response is seen in SD male rats, however.

A two-year bioassay in both sexes of the mouse was negative for carcinogenicity, as were two-year bioassays in male and female F-344 rats (Hazeltte and Green, 1987; Thakur, 1991b; Thakur, 1992b).

Table 5-1 displays summaries of the mammary tumor incidence and onset in all the rodent bioassays that have been submitted to the Agency and also a study from the open literature (Pinter *et al.*, 1990). Additional details concerning these studies can be found in the discussion that follows. Appendix Table 1 also summarizes in further detail the results from the studies performed in the SD rat.

Table 5-2 displays summaries of pituitary adenoma incidences in all the rodent bioassays that have been submitted to the Agency. Pituitary tumor onset is difficult to determine as pituitary tumors are not palpable as are mammary tumors. However, in a serial sacrifice study, an early onset of pituitary tumors can be discerned in female SD rats (Thakur, 1991a). Only female pituitary tumor incidence is displayed in Table 5-2.

[1]Data from the studies referred to here as Thakur, 1991a, Thakur, 1991b, Thakur 1992a and Thakur 1992b, have been published in the open literature as Wetzel *et al.* 1994.

Table 5-1. Summary of Female Mammary Tumor Incidence in Two- and One-Year Rodent Bioassays Using Atrazine

Study	Species/ Strain	Duration	Mammary Tumor Incidence	Mammary Tumor Onset
Mayhew *et al.*, 1986	Rat/SD	2 year	Statistically-significant increase in female carcinomas at 3.5 mg/kg/day when adjusted for survival	Not determined in this study
Thakur, 1991a	Rat/SD	2- year with serial sacrifices	A significant positive trend for fibroadenomas is seen.	The percentage of carcinomas occurring in the first year of the study was 0 in controls, 33% at 4.23 mg/kg/day, and 50% at 26.23 mg/kg/day.
Thakur, 1992a	Rat/SD	2- year	No statistically-significant increases in female fibroadenomas or carcinomas seen at either 3.79 or 24.01 mg/kg/day	The percentage of carcinomas and adenomas occurring in the first year of the study in controls was 0% while at 3.79 mg/kg and 23.01 mg/kg/day 27.3 and 33.3% of the carcinomas appeared in the first year of the study.
Morseth, 1998	Rat/SD, both OVX and intact	2-year	No tumors seen in OVX animals. Carcinoma, and fibroadenoma incidences at 3.1 mg/kg/day are increased two-fold over control values in intact animals.	The mean week of onset for carcinomas and adenomas in controls was 72.6 while the mean week of onset for the 1.5, 3.1, 4.2 and 24.4 mg/kg/day groups was 77.2, 78.6, 64.4 and 64.8.
Pettersen and Turnier, 1995	Rat/SD	1-year	six carcinomas/adenomas and four fibroadenomas are seen at the 23.9 mg/kg/day group compared to one carcinoma and two fibroadenomas in the control group.	The increased incidence of tumors at one year indicates an earlier onset.
Hazelette and Green, 1987	Mouse/CD-1	91 weeks	No increase in any tumor in either sex with exposures up to 386 mg/kg/day for males and 483 mg/kg/day for females	Not altered in Atrazine exposed animals
Thakur, 1991b	Rat/F-344	2- year with serial sacrifices	No increase in any tumor in either sex with exposures up to 34 mg/kg/day in both	Not altered in Atrazine exposed animals
Thakur, 1992b	Rat/F-344	2-year	No increase in tumors of any kind in either sex with exposures up to 20 mg/kg/day for males and 26 mg/kg/day for females	Not altered in Atrazine exposed animals
Pinter *et al.,* 1990	Rat/F-344	Lifetime	Statistically-significant increase in male benign mammary tumors	Increased survival in dose groups versus controls resulted in delayed time of onset

Table 5-2. Summary of Female Pituitary Adenoma Incidence in Two- and One -Year Rodent Bioassays Using Atrazine

Study	Species/ Strain	Duration	Pituitary Adenoma Incidence by Dose Group (doses in mg/kg/day)
Mayhew *et al.*, 1986	Rat/SD	2 year	Control= 47/68 (69%); 0.5 = 41/63 (65%); 3.5 = 49/68 (72%); 25= 47/65 (72%); 50= 35/63 (56%)
Thakur, 1991a	Rat/SD	2- year with serial sacrifices	Control = 22/70 (31%); 4.23 = 16/70 (23%); 26.23 = 20/70 (29%)
Thakur, 1992a	Rat/SD	2- year	Control = 43/58 (74%); 3.79 = 45/58 (78%); 23.01 = 46/60 (77%)
Morseth, 1998	Rat/SD, both OVX and intact	2- year	*OVX* - Control = 42/80 (53%); 1.5= 39/80 (49%); 3.1 = 35/80 (44%); 4.2 = 42/80 (53%); 24.4 = 41/80 (51%) *Intact* - Control = 56/80 (70%); 1.5= 60/80 (75%); 3.1 = 52/80 (65%); 4.2 = 56/80 (70%); 24.4 = 54/80 (68%)
Petersen and Turnier, 1995	Rat/SD	1-year	Control= 2/55 (4%); 0.8 = 5/55 (9%); 1.7 =6/55 (11%); 2.8 = 4/55 (7%); 4.1 = 1/55 (2%); 23.9 =5/55 (9%)
Hazelette and Green, 1987	Mouse/CD-1	91 weeks	Control= 0/60; 1.6 = 0/60; 47.4 = 0/60; 246.9 = 3/60 (5%); 482.7 = 0/60
Thakur, 1991b	Rat/F-344	2- year with serial sacrifices	Control= 9/67 (13%); 0.68 = 6/69 (9%); 4.82 = 7/65 (11%), 14.05 = 5/66 (8%); 34.33 = 5/67 (7%)
Thakur, 1992b	Rat/F-344	2-year	Control = 22/60 (37%); 0.49 = 26/60 (43%), 3.43 = 20/58 (34%); 9.87= 19/59 (32%); 20.17 = 13/59 (22%)
Pinter *et al.,* 1990	Rat/F-344	Lifetime	Control = 32/41 (78%); 18.75 = 23/43 (53%); 37.5 = 35/50 (70%)

5.1 Mayhew *et al.*, 1986

The initial study that raised concerns about the possible carcinogenic effects of atrazine exposure was a carcinogenicity study conducted in male and female Sprague-Dawley rats at dietary dose levels of 0, 10, 70, 500 or 1000 ppm (0, 0.5, 3.5, 25 or 50 mg/kg/day). The Maximum Tolerated Dose (MTD) was likely exceeded in this study at the 1000 ppm dose in females. Mortality was significantly increased from 49% mortality at 104 weeks in the controls to 75% mortality at 104 weeks in females of the 1000 ppm group (see Table 5-4). Terminal body weight was also significantly decreased in 1000 ppm females in this study. There was a 27.2% decrease in group mean body weight ($p<0.01$) in the 1000 ppm females compared to controls. Male survival in the 1000 ppm group was significantly increased at 1000 ppm compared to controls, but body weight was significantly decreased compared to controls - 18.7% less than controls at 104 weeks. Based on the decreased body weight and increased mortality seen in females, and the decreased body weight seen in males, 1000 ppm is deemed to exceed the MTD of Atrazine in this strain of rats. The second-highest dose in this study, 500 ppm, likely is very close to the MTD. Male body weight at this dose is reduced 8.2% compared to controls at 104 weeks while male survival is not significantly altered compared to controls. Female survival is not significantly altered at this dose compared to controls, but body weight is reduced by 18.9% ($p<0.05$) compared to controls at 104 weeks. Based on the lack of significant effect in males seen at the 500 ppm dose and the uncertain effect seen in females (lack of a significant increase in mortality with a significant decrease in body weight) it seems likely that 500 ppm is very close to the MTD for atrazine in this strain of rat.

The conclusions drawn about the MTD of atrazine are important given that 1000 ppm exceeds the MTD and 500 ppm is assumed to be very close to the MTD. Thus, subsequent two-year carcinogenicity studies have used a dose of 400 ppm as the high dose to have the high dose be slightly below the MTD.

The mammary tumor incidences seen in this study are reported below in Table 5-3 and mortality is shown in Table 5-4.

Table 5-3. Mammary Tumor Incidence in the Mayhew Study (as determined by US EPA, 1988)

Tumor Type	Dose (mg/kg/day)				
	Control	0.5	3.5	25	50
adenocarcinomas/ carcinosarcomas combined	15/88 17% 0.000**	16/67 24% 0.39	27/69 39% 0.024*	27/68 40% 0.019*	45/60 51% 0.000**
adenomas and fibroadenomas combined	20/88 23% 0.446	24/65 37% 0.110	21/69 30% 0.373	21/68 31% 0.373	20/89 22% 0.468

NOTE: Significance for the trend is indicated at control. Significance of pairwise comparison *vs.* controls is noted at dose group.

Incidence values are number of tumor bearing animals over number of animals at risk

*[1]p< 0.05; **p<0.01 as indicated by Peto Prevalence Test

Table 5-4. Mortality in the Mayhew Study (as determined by US EPA, 1988)

	Dose (mg/kg/day)				
	Control	0.5	3.5	25	50
Mortality at terminal sacrifice	34/59 49%**	39/70 56%	40/70 57%	44/70 63%	52/69 75%**

NOTE: Significance for the trend is indicated at control. Significance of pairwise comparison noted at dose group. Statistical test used are cox's or Generalized Krushkal-Wallis.

**p<0.01

A type of statistical analysis that examines tumor incidence and mortality is the Peto Prevalence test. The results of this test are displayed in Table 5-3. The results of this test showed that for the 70, 500 and 1000 ppm groups there was a statistically-significant (SS) pairwise increase in incidence of mammary adenocarcinomas and carcinosarcomas combined at 70, 500 and 1000 ppm, and that there was a dose-related trend for these tumors that was also SS ($p < 0.01$). The study authors of the Mayhew report also conducted Cox-Tarone and Gehan-Breslow tests to examine tumor incidence in light of the decrease mortality in the females. The results from these tests were similar to the results from the Peto test.

5.2 Thakur Studies

These are four studies - two using the SD strain and two using F-344 strain. These studies consisted of both terminal (all animals sacrificed after two-years exposure) and serial sacrifice (10 animals per group sacrificed at varying timepoints) protocols. The studies using the SD strain are discussed below while the studies with the F-344 strain are discussed in section 5.6 Appendix Table 2 displays summaries of the study design for these studies.

5.2.1 Serial Sacrifice Protocol (Thakur, 1991a)

Seventy SD female rats (no males were used) were exposed through the diet to doses of atrazine (97%) at 0, 70 and 400 ppm (0, 4.23 and 26.23 mg/kg/day) for two years. Ten females per dose were sacrificed at one, three, nine, 12, 15, 18 and 24 months.

Mortality was increased in a dose-dependent manner. There were five unscheduled deaths in the control group, six in the 70 ppm group, and eight at 400 ppm. Using the Gehan-Breslow test there was a statistically- significant (SS) negative trend for survival (survival decreased as the dose increased). Another statistical test - the Cox-Tarone test - did not indicate a significant trend in either direction. A statistically-significant reduction in body weights were found at several timepoints in the 400 ppm group compared to controls.

The results of this study is significant in regards to pituitary tumors. Because pituitary tumors cannot be detected by palpation, a serial sacrifice study is the most appropriate way to determine onset for pituitary tumors. Pituitary tumor incidences by timepoint are displayed in Table 5-5, below. The hormonal basis for early onset of pituitary tumors is discussed in section 9.2.6

Table 5-5. Pituitary â-Adenoma Incidences by Timepoint in Thakur Serial Sac, 1991a

Sacrifice time (mo.)	Control	4.23 mg/kg/day	26.63 mg/kg/day
1	0	0	0
3	0	0	0
9	0	0	2
12	2	2	6
15	5	3	4
18	9	5	6
24	6	6	2
0-12	2	2	8
0-24	22	16	20

NOTE: Ten animals in each group. Unscheduled sacrifice animals are included in this table

5.2.2 Terminal Sacrifice Protocol (Thakur, 1992a)

Sixty SD females (no males were used) were exposed through the diet to doses of atrazine of 0, 70 and 400 ppm (0, 3.79 and 23.01 mg/kg/day) for two years (Appendix Table 2 summarizes the protocol used for the Thakur studies with both SD and F-344 rats). Mortality was high in the controls and increased in a dose-related manner. Mortality was 48% in controls, 58% in the 70 ppm group, and 63% in the 400 ppm group. Two survival tests were contradictory in determining whether or not this increase in mortality was significant. Analysis with the Gehan-Breslow test showed a negative trend in survival with dose (increased mortality with increasing dose) while a Cox-Tarone test found that the increases in mortality were not significant. Group mean body weights were significantly decreased, compared to controls, at the 400 ppm group as early as four weeks and remained significantly decreased up to, and including, week 76. Body weight gains were significantly decreased for the period from study initiation to week 76. Both absolute body weight at week 104 and body weight gain from week five to 104 of the 400 ppm group, were lower than controls, but not statistically-significantly. Group mean food consumption in the 400 ppm group was decreased compared to controls for the first 13 weeks of the study. After 13 weeks though, there was no significant difference. The only finding at gross necropsy that may have been related to compound exposure was an increase in enlarged spleens in the 400 ppm dose group. The control and 70 ppm groups were observed to have five and three animals with enlarged spleens, respectively, while the 400 ppm group had 15. This finding was not observed in the Mayhew study, in the Thakur terminal sacrifice study, or in any other bioassays that followed the Thakur series of studies.

Histopathology revealed that mammary and pituitary neoplasms were a common occurrence. Table 5-6 displays mammary tumor incidence data by tumor type. There was not a statistically-significant increase in fibroadenomas or carcinomas at the doses tested, compared to controls. This is true whether or not mortality is taken into account through Cox or Gehan-Breslow tests. Table 5-6 displays the p values calculated by the study authors for mammary tumor incidences.

Table 5-6. Female Mammary Gland Tumor Incidences in

the SD Terminal Sacrifice Protocol (Thakur, 1992a)
(calculated using Cox-Tarone and Gehan-Breslow tests)

	Dose (mg/kg/day)		
	Control	**3.79**	**23.01**
Fibroadenoma	39/60 (65%)	30/59 (51%)	41/60 (68.3%)
p value			
Cox-Tarone		0.9141	0.1070
Gehan- Breslow		0.6401	0.2114
Carcinoma	17/60 (28%)	13/59 (22%)	22/60 (33.6%)
p value			
Cox-Tarone		0.8316	0.1590
Gehan- Breslow		0.7613	0.0810

5.3 Morseth, 1998

Atrazine (97.1%) was administered to 800 female Sprague-Dawley rats. The rats were divided into two groups of 400 each. One group was ovarectomized (OVX) while the other was left intact. Atrazine was mixed with the diet at dose levels of 0 (control) 25, 50, 70 and 400 ppm (0, 1.5, 3.1, 4.2, 24.4 mg/kg/day for intact animals and 0, 1.2, 2.5, 3.5, and 20.9 mg/kg/day for OVX animals) for two years. There were 80 females at each dose level - 20 for a 12-month sacrifice and 60 for a 24-month sacrifice.

The trend for survival was statistically-significantly (SS) decreased in the dosed groups compared to the controls. Survival was as follows: 43.3% in controls; 31.7% - 25 ppm; 28.8% - 50 ppm; 31.6% - 70 ppm; 21.7% 400 ppm. Body weight was SS reduced in the first half of the study in the 400 ppm group (other groups were not significantly altered), but by the end of the study body weights were similar to control values.

Neoplastic histopathological findings were mostly limited to the pituitary and the mammary gland. Neither intact nor OVX dosed animals showed an increase in pituitary tumors compared to their respective controls, but intact animals did show a 20-30% greater incidence of pituitary adenomas compared to OVX animals.

There were few mammary tumors in the interim sacrifice animals, which is not surprising given that these animals were sacrificed after only one-year. Excluding the interim sacrifice and looking only at those animals that were sacrificed at 24 months and those that died prematurely, there was an increase in mammary tumor incidence at all intact dose groups compared to controls. Looking at carcinomas alone incidence values are: 18.3%; 36.7%; 33.9%; 20%; and 41.7% for the control, 25, 50, 70 and 400 ppm dose groups respectively. Fibroadenomas alone were: 26.6%; 40%; 52.5%; 45%; and 40% for the control, 25, 50, 70 and 400 ppm dose groups respectively.

Table 5-7 below displays a statistical analysis of mammary tumor incidence in intact animals using a Peto's Prevalence Test. Incidence values shown below differ from those described in the paragraph above because interim sacrifice animals are included in the analysis shown in Table 5-8 (USEPA, 1999c). A different survival-adjusted statistical analysis (Cox-Tarone) conducted by the study author showed significant pairwise increases at 3.1 mg/kg/day for fibroadenomas compared to control, but did not show significant pairwise comparison to control for the 1.5 mg/kg/day group.

Table 5-7. Mammary Gland Tumor Incidence in Intact Animals in Morseth, 1998 Study

	Dose (mg/kg/day)				
	Controls	**1.5**	**3.1**	**4.2**	**24.4**
Fibroadenoma	16/78 (21%) 0.233	25/79 (32%) 0.030*	34/77 (44%) 0.000**	29/78 (37%) 0.014*	25/77 (32%) 0.014*
Carcinoma	12/80 (15%) 0.002**	18/80 (22%) 0.112	20/79 (25%) 0.067	14/80 (18%) 0.395	27/80 (34%) 0.007**
Adenoma	0/28	0/24	1/20 (5%)	0/21	0/15

NOTE: Significance for the trend is indicated at control. Significance of pairwise comparison noted at dose group. Incidence values are number of tumor bearing animals over number of animals at risk.

*p<0.05; **p<0.01

Not a single mammary tumor of any sort was seen in any OVX animal. The lack of mammary tumors in OVX animals provides evidence indicates that an intact ovary is mandatory for mammary tumorogenesis in the SD female. The results found in OVX animals will be discussed more fully in section 7.3. Bi-weekly estrous cycle measurements were also made in this study. The results of these measurements are discussed below under section 9.2 Estrous Cycle.

5.4 Pettersen and Turnier, 1995

This study exposed female SD rats (55 per group) through the diet to doses of atrazine of 0, 15, 30, 50, 70, or 400 ppm (0, 0.8, 1.7, 2.8, 4.1, or 23.9 mg/kg/day). This study was a serial sacrifice protocol in which 10 animals in each group were sacrificed at 3, six, and nine months and the remaining 25 animals were sacrificed at one year following initiation of dosing. Dosing appeared to be adequate, as body weights in the last 10 months of the study were reduced 8 to 12% in the 400 ppm animals compared to controls. Body weight gains over the last 10 months of the study were reduced 11 to 18% in the 400 ppm group compared to controls. There were no differences in survival among dose groups in this study. No mammary tumors were seen in the three- and six-month sacrifices. There was a significant positive dose-related trend in mammary tumors as well as a significant increase in mammary tumors between control and 400 ppm animals using a pairwise comparison. Table 5-8 displays tumor incidences from this study.

Table 5-8. Number Of Animals With Mammary Tumors In The Pettersen and Turnier, 1995 Study

	Dose(mg/kg/day)					
	0	**0.8**	**1.7**	**2.8**	**4.1**	**23.9**
3 and 6 months	0/20 had tumors	0/20 had tumors	0/20 had tumors	0/20 had tumors	0/20 had tumors	0/20 had tumors
9 month	F=1/10	C=1[1] no other tumors	0/10 had tumors	0/10 had tumors	F=1/10	C=1/10 F=1/10
12 month (Terminal Sac)	C=1/25 F=1/25 A=0/25	C=1/24 F=2/24 A=0/24	C=0/25 F=2/25 A=1/25	C=1/25 F=0/25 A=1/25	C=1/24 F=3/24 A=1/24	C=5/25 F=3/25 A=1/25
Total	C/A=1/55 F=2/55	C/A=2/55 F=2/55	C/A=1/55 F=2/55	C/A=2/55 F=0/55	C/A=2/55 F=4/55	C/A=7/55 F=4/55

NOTE: Numerator is the number of animals with tumors, denominator is the number of animals examined

C=adenocarcinoma; F= fibroadenoma; A = adenoma

[1]This tumor occurred in an animal scheduled to be sacrificed at 12 months but found dead on study day 218.

5.5 Hazelette and Green, 1987

Atrazine (purity not given) was administered to CD-1 mice through the diet to 59-60 animals/sex/dose, at dose levels of 0,10,300,1500 and 3000 ppm (male/female mean daily dose 0/0, 1.4/1.6, 38.4/47.9, 194.0/246.9, 385.7/482.7 mg/kg/day)for 91 weeks. The doses given were adequate as indicated by toxic effects, such as a decrease in mean body weight gain of both sexes (23.5%/11%, M/F) and an increase in cardiac thrombi in the females, are seen at both 1500 and 3000 ppm, while no dose-related toxic effects are seen at 10 and 300 ppm. There was also an increase in mortality ($p < 0.05$) in 3000 ppm females, but not males, with only 25% of the females surviving versus 39-43% of the females surviving in the other female dose groups. At the doses tested, there was not a treatment-related increase in tumor incidence when compared to controls.

5.6 F-344 Two-Year Bioassays

5.6.1 Serial Sacrifice Protocol (Thakur, 1991b)

Seventy F-344 rats (females only) per dose were exposed ad libitum to diet that had been mixed with atrazine (97.1%) to the appropriate doses of 0 (negative control), 10, 70, 200 and 400 ppm (0, 0.68, 4.82, 14.05, 34.33 mg/kg/day). Ten animals per dose group were sacrificed after approximately one, three, nine, 12, 15, and 18 months exposure to the test article.

There was not an increase in mortality due to compound exposure, and there was no increased incidence of clinical signs in dosed animals compared to controls. The doses tested appeared to be sufficiently high because there was a decreased absolute body weight and body weight gain in the 400 ppm group compared to the controls. Group mean absolute body weight in the 400 ppm group compared to controls was also significantly decreased compared to controls at several time points though the final mean body weight was not significantly decreased compared to controls. The final group mean body weight for the 400 ppm group was 6.6% less than the mean control value. During the course of the study the 400 ppm animals gained an average of 116.7 gm compared to the weight gain in the control group of 133.3 gm (14% less than controls). This difference in body weight gain was statistically-significant at a p value of 0.05. There was not an increase in mammary tumors or any other type of tumor at any dose group.

5.6.2 Terminal Sacrifice Protocol (Thakur, 1992b)

Sixty F-344 rats per sex per dose were fed technical grade atrazine through the diet at doses of 0 (negative control), 10, 70, 200 and 400 ppm (0, 0.49, 3.43, 9.87 and 20.17 mg/kg/day for males and 0, 0.61, 4.35, 12.71, and 26.18 mg/kg/day for females) for two years. Mortality in either sex was not affected by treatment. Male control mortality was 30% while male mortality in the 400 ppm group was 32%. Mortality in the other male dose groups was slightly lower than controls, ranging from 22 to 25%. Female mortality was 22% in the controls and 27% in the 400 ppm group. Female mortality in the other dose groups ranged from 17 to 25%. Body weights and body weight gains were adversely affected by compound exposure, especially at the 400 ppm dose in each sex. Mean group body weights were statistically-significantly reduced versus controls at the four, 13, 24, 52, 76 and 104 week timepoint in the both the male and female 400 ppm group. Percent body weight reductions ranged from 5.1 to 9.3% in the males and 5.3 to 6.4% in the females. Percent body weight gains were also significantly decreased in both sexes of the 400 ppm group for all the time periods examined - 0-4, 0-13, 0-24, 0-52, 0-76 and 0-104 weeks. The range of percent reductions compared to controls was 11.3 to 15.9% in males and 10.7 to 17.4% in females. The reduction in percent body weight gain, compared to controls, in males for the entire study (weeks 0-104) was 11.3% and for females it was 11.6%. Mean group food consumption was significantly decreased (4.8% versus controls) for the 0-104 week period in 400 ppm males, but was not significantly decreased in females. There were no findings at gross necropsy that could be attributed to compound exposure and organ weights were not altered between control and dosed animals.

The incidence of mammary gland fibroadenomas was increased in dosed females compared to controls. This increase was not statistically-significant. Even at its highest dose level the percentage of animals with fibroadenomas was below the historical level for the laboratory where the Thakur studies were conducted. There were no increases in mammary tumors of any type in dosed males versus controls. Two out of the 55 control males examined at histopathology were found to have a mammary tumor (both were fibroadenomas). Only one out of 54 males in the 10 ppm group and one out of 58 in the 400 ppm group were found to have mammary tumors (one fibroadenoma and one carcinoma) while not males in the 70 and 200 ppm group had a mammary tumor of any type. Therefore, dosing with atrazine did not increase mammary tumor incidence in F-344 males.

Table 5-9. Female Mammary Tumor Incidence In F-344 Terminal Sacrifice Protocol In The Thakur Terminal Sacrifice Study (1992b)

	Dose (mg/kg/day)					
	Control	**0.5**	**3.4**	**9.9**	**20.2**	**Historical Control**
Fibroadenoma	2/60 (3.3%)	5/60 (8.3%)	5/60 (8.3%)	7/60 (11.7%)	6/59 (10.2)	Mean = 14.9% Range= 3-23%
Unadjusted p value	0.2514	0.2198	0.2195	0.0815	0.1295	
Carcinoma	2/60 (3.3%)	0/60 (0%)	2/60 (3.3%)	3/60 (5%)	2/59 (3.4%)	Mean = 3.8% Range= 2-15%
Unadjusted p value	0.4640	0.2479	0.0907	0.5000	0.6843	

NOTE: Historical control data from Hazelton Labs, 1984

5.7 Pinter *et al.*, 1990

The Pinter *et al.* study exposed Fischer-344 rats of both sexes to atrazine (98.9%) that was mixed in the diet at concentrations of 0, 500 and 1000 ppm. The control groups started with 56 males and 50 females; the 375/500 ppm group started with 55 males and 53 females; and the 750/1000 ppm group started with 53 males and 55 females. Unlike most carcinogenicity assays where surviving animals are sacrificed after approximately 104 weeks on study, in this study animals were allowed to live out their natural life span, except for four males and six females that were sacrificed moribund. Table 5-10 displays the mammary tumor incidence in males in Pinter *et al.*, 1990.

Table 5-10. Mammary Tumors In Males in Pinter *et al.*, 1990 [1]

	Control	375 ppm	750 ppm
Total number of tumor-bearing males	1/48	1/51	8/53
Total number of benign tumors	1/48	1/51	9/53**
Total number of malignant tumors	0/48	0/51	1/53

[1]Data from Table 2 in Pinter *et al.,* 1990.

**p<0.01 using Fisher Exact test comparing high dose group to low dose group.

5.8 Pinter *et al.*, 1990

The Pinter *et al*. study exposed Fischer-344 rats of both sexes to atrazine (98.9%) that was mixed in the diet at concentrations of 0, 500 and 1000 ppm (the 500 and 1000 ppm doses were reduced to 375 and 500 ppm after eight weeks of treatment due to toxicity). The control groups started with 56 males and 50 females; the 375/500 ppm group started with 55 males and 53 females; and the 750/1000 ppm group started with 53 males and 55 females. Unlike most carcinogenicity assays where surviving animals are sacrificed after approximately 104 weeks on study, in this study animals were allowed to live out their natural life span, except for four males and six females that were sacrificed moribund. Table 5-10 displays the mammary tumor incidence in males in Pinter *et al*., 1990.

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	Control	375 ppm	750 ppm
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Total number of benign tumors	1/48	1/51	9/53**
Total number of malignant tumors	0/48	0/51	1/53

[1]Data from Table 2 in Pinter *et al.,* 1990.

**p<0.01 using Fisher Exact test comparing high dose group to low dose group.

Mammary gland tumors in the dosed females in this study were not altered in incidence compared to controls. Mammary gland tumors in dosed males were altered. The incidence of benign tumors (adenomas, fibroadenomas, and fibromas) was one tumor in 48 animals in controls, 1/51 in the 375 ppm group and 9/53 in the 750 ppm group. The study authors performed a statistical analysis to determine if this increase in tumors was significant. The authors found that when a pairwise comparison was done between the 750 ppm group and the 375 ppm group there was a significant increase at 750 ppm ($p<0.01$). Generally dose groups are compared to controls to determine changes in tumor incidence following dosing. The study authors choose not to perform a pairwise comparison to controls in this case, however, because the animals in the control group died much sooner than the animals in either dose group. The last control male died before study week 120; the last 375 ppm male died between week 120 and 130; and the last 750 ppm male died between weeks 130 and 140. The differences in survival in this study confound the results. The study authors state: "The tumors in the high-dose group appeared later in time than those in the control or low-dose group." The one tumor in the control group occurred at week 111 and the sole tumor in the 375 ppm group occurred at 119 weeks. By contrast, the average mean time of tumor appearance in the 750 ppm group was 121.3 week ± 15.4 weeks. The increase in tumors seen in the older animals could be due to the exposure to atrazine or could also be due to the simple fact that these were old animals.

Mammary tumors in F-344 males have been shown to increase in

incidence in untreated males as they age (Solleveld, 1984). Table 5-11 displays mammary tumor incidences in untreated, aged F-344 males. Table 5-11shows that a difference of only a few months can greatly increase the incidence of mammary tumors as the rats age. Tumor incidences double as the rats age from 98-110 weeks to 111-123 weeks. Incidences double again as the rats age from 111-123 weeks to 124 to 136 weeks.

Table 5-11. Mammary Gland Fibroadenomas in Male F-344 Rats by 12 Week Time Periods (Solleveld, 1984)

	98-110 Weeks	111-123 Weeks	124-136 Weeks	>137 Weeks
Mammary Fibroadenoma	3/77 (4%)	13/143 (9%)	27/148 (18%)	22/95 (23%)

The study authors seem to realize the possible relationship between the age of the high-dose males and their high tumor incidences. The authors cite Solleveld (1984) in an attempt to show that, even given the increased tumor incidence in aged males, the atrazine exposed males had increased tumor incidences. Pinter *et al.*, (1990) notes:

> "The incidence of benign mammary gland tumors in male F344 rats was reported to be 2.2% for 110 to 16 weeks; in the life span studies (more than 116 weeks), however, 13.4% of the male animals had benign mammary gland tumors [Solleveld, 1984 is cited]. In our study, 16.9% of the high-dose, males had benign mammary tumors."

The 2.2% the authors of the Pinter *et al.* study refer to in the above quote is historical control data from untreated males in several National Toxicology Program two-year bioassays. The 13.4% is historical control data for mammary tumors in untreated males greater than 116 weeks in age in life-span studies. The difference between 2.2% mammary tumor incidence at 116 weeks in the two-year bioassays and the 13.4% in the life span studies again emphasizes the dramatic increases in tumors that occur as the animals age beyond approximately two years of age.

The study authors seem to believe that atrazine exposure is inducing the additional tumors between 13.4% and 16.9%. However, the study author's Table 2 on page 537 of their publication shows that only eight of 53 (15%, not 16.9%) males had a mammary tumor of any sort. The origin of the 16.9% value is unknown. The true difference - according to the data the study authors present in their Table 2 - is between the 13.4% and 15%. Additionally, there was one incidence of an adenocarcinoma in the high-dose males. The Pinter *et al*. publication does not include any description of which male had this carcinoma but if this tumor occurred in an animal that did not also have a benign tumor then the number of animals with benign tumors drops to seven out of 53 -- 13.2% -- almost identical to the 13.4% cited by the study authors from the Solleveld (1984) paper.

It is concluded that the authors of this study have not made a case that the increase in male benign mammary tumors is due to atrazine exposure. The tumors appearing in the high-dose males do not appear to be found at a rate any higher than what would be expected for F-344 males of a comparable age.

5.9 Summary and Discussion of the Two-Year Bioassay Studies

Increased incidences of mammary fibroadenomas or carcinomas were seen in three out of four separate two-year bioassay studies using Sprague-Dawley rats.

Atrazine exposure in a two-year bioassay using CD-1 mice did not result in increased incidences of mammary tumors in either sex, despite the compound being given to the mice at doses that resulted in decreases in body weight gain of 23.5% in females and 11% in males and a significant increase in mortality in females.

Atrazine exposure in two separate two-year bioassays using the F-344 strain of rats also did not result in increased incidences of mammary tumors. One of the bioassays used rats of both sexes (terminal sacrifice protocol) and did not see increases in mammary tumors in either sex, while the other study employed only females (serial sacrifice protocol) without seeing an increased incidence of mammary tumors. Dosing was adequate in both studies as indicated by the 14% decrease in female body weight gain the 400 ppm dose group compared to controls (serial sacrifice protocol) and the 11.3% (♂) and 11.6% (♀) reductions in percent body weight gain at 400 ppm compared to controls in the terminal sacrifice study. Doses of atrazine that produced equivalent, or even less than, reductions in body weight in the two-year bioassays with the SD strain reductions) produced mammary tumors. For example, in Thakur, 1992a (the terminal sacrifice protocol), female body weights gains were reduced 12 to13% at 104 weeks in the 400 ppm group; in Mayhew *et al*. (1986) female body weight in the 70 ppm group were reduced only 3.25, yet this was still sufficient to produce mammary tumors.

A study by Pinter *et al.* (1990) also showed a lack of carcinogenic effect for atrazine in F-344 females. This study did find an increase in benign mammary tumors in F-344 males when high-dose males were compared to low-dose males. However, this study was a life-span study rather than a two-year bioassay and the males of the high-dose group (in which the increases in benign mammary tumors were seen) survived significantly longer than males in the controls or low-dose group. Examination of the tumor incidence in the high-dose males from this study suggest that mammary tumor incidences were most likely comparable to what would be expected for males of this strain and age.

Chapter 6

6 Genotoxicity Studies

An important question to address in the hazard assessment is whether atrazine also has the potential to be DNA reactive and act as an initiator within the context of the multistage model of carcinogenesis. Thus, following initiation (*i.e.*, a mammary gland cell acquires a mutation that results in unregulated proliferation), tumor promotion (*i.e.*, clonal expansion of the genetically altered cell) in the mammary tissue would then be hormonally-mediated. The most desirable data to address this issue would be information on genetic alterations in the relevant target tissue. This information is not available for atrazine. There are a large number of studies using standard genotoxicity assays to evaluate the mutagenic potential of atrazine.

Atrazine has been examined for its ability to induce mutations in microorganisms, insect, and plants, and to induce chromosomal aberrations *in vitro* and *in vivo* in both mammalian and nonmammalian organisms. Additionally, atrazine has been tested in other assays using endpoints that are indicative of DNA damage, but are not measures of mutation *per se* (*e.g.*, genetic recombination, sister chromatid exchanges, DNA strand breakage, and unscheduled DNA synthesis).

Although more than 50 studies are available on atrazine, some findings reported in the literature are presented in insufficient detail for evaluation or results are inconclusive due to study design problems. Furthermore, the results on atrazine are inconsistent even within the same test system and genetic endpoint evaluated. Thus, in evaluating the mutagenic potential it is important to take a weight- of- evidence approach that considers the overall response patterns or trends for mutation, chromosomal damage, and other indicators of DNA damage. In looking at the overall trends in the database, it is important to consider the type of end point evaluated and the test system/organism used. More emphasis is placed on end points that are direct measures of mutation and chromosomal aberrations rather than indicators of DNA damage (*e.g.*, sister chromatid exchanges, DNA strand breaks). Also, results from mammalian systems are emphasized more than results from assays using nonmammalian organisms. Likewise, mammalian *in vivo* data are preferred over data from *in vitro* tests.

As summarized in Appendix Table 5 Most of the mutagenicity studies on atrazine have been reported as negative. The majority of these negative results come from mutation studies in bacteria. Beyond the bacteria results, the response profile for atrazine is heterogenous and closer to a split between negatives and positives. Nevertheless, the response patterns or trends for mutation and chromosomal damage tend to be more convincing for the number and type of negative responses found after atrazine treatment than for the positive data, which typically were weak, observed at high treatment concentrations of atrazine, or were not repeatable. Data on several metabolites of atrazine and its close structural analogues (propazine and simazine) do not support a mutagenic potential for these compounds. Therefore, the totality of evidence does not support a mutagenic potential for atrazine, and indicates that a direct DNA reactive/mutagenic mode of action is unlikely to be an influence of atrazine on mammary gland tumor development (or at any other site). A discussion of the literature supporting this conclusion follows.

6.1 Mutation Studies

As summarized in Appendix Table 5, most studies on atrazine for mutation induction are bacteria tests with a few assays in yeast, fungi and in the fruit fly *Drosophila melanogaster*. There is no compelling evidence for mutation induction as a mode of carcinogenic action for atrazine given the consistent negative responses in bacterial tests, and the inconsistent positive responses across other phylogenetic lines (where responses tended to be weak, found at high doses, and/or were not reproducible).

When atrazine was evaluated in the Ames assay (with a variety of *Salmonella typhimurium* tester strains) by several different laboratories, it was consistently negative even when a mammalian liver metabolic activation system was incorporated (Seiler, 1973; Poole and Simmon *et al.*, 1977; Lusby *et al.*, 1979; Bartsch *et al.*, 1980; Sumner *et al.*, 1984; Deparde, 1986; Kappas,1988; Mersch-Sundermann *et al*., 1988; Zeiger *et al*., 1988 1992; Ruiz and Marzin, 1997).

There are no acceptable mutation studies in: Butler and Hoagland, 1989; Anderson *et al.*, 1972; Morichetti *et al.* 1992, mammalian systems. Although Adler (1980) reported a negative result for a gene mutation test (HPRT assay) in V79 cells, this paper does not contain sufficient detail to allow an independent assessment of the finding.

Tests in yeast and fungi have yielded heterogenous results. For example, mutation induction was reported in *Schizosaccharomyces pombe* with or without plant cell activation (Mathias, 1987) and in *Aspergillus nidulans* only with mammalian cell activation (Benigni *et al.*, 1979). When atrazine was evaluated in *Saccharomyces cerevisiae* without exogenous activation, a negative result was reported in one paper (Emnova *et al.*, 1987), while a weak positive finding was observed by Morichetti *et al.*, 1992. The reported positives are mostly found at high doses of atrazine. Furthermore, gene conversion and mitotic recombination, which are indicators of DNA damage, were not increased in yeast and fungi exposed to atrazine (de Bertoldi *et al.*, 1980; Emnova *et al.*, 1987; Kappas, 1988), except when plant cell activation was incorporated into the assay (Plewa and Gentile, 1976). Because these lower eucaryotic assays have intrinsic rates of positive responses that occur sporadically, this conflicting database in fungi and yeast must be interpreted carefully in the context of the weight-of- evidence and results from other organisms.

Microbial systems (*Salmonella, E. coli*, yeast) have been used as indicators of mutational damage after atrazine treatment in host-mediated assays (Adler, 1980; Simmon *et al.*, 1977). The mouse host-mediated assays on atrazine have yielded mix results, with *Salmonella* (injected intraperitoneally) being negative and *E. coli* and yeast (injected into the mouse testes) as positive. However, because of the variability in cell recovery, these assays are not viewed as reliable indicators of mutagenicity.

Some information is available in insects. In general, positive results in *Drosophila* were reported for somatic mutation in the spot wing test (Tripathy *et al*., 1993; Torres *et al*., 1992) or in the sex-linked recessive lethal assay (Tripathy *et al*., 1993; Murnik and Nash, 1977) under certain conditions (larval feeding or at high doses). Murnik and Nash (1977) tested atrazine, simazine and cyanazine in the *Drosophila* sex linked recessive lethal assay. The authors further concluded that, "these triazine herbicides may be weak mutagens," and that "Much larger experiments are needed to determine with confidence the mutagenic potential of the herbicides." The results reported by Murnik and Nash (1977) were considered inconclusive by an expert Gene-Tox panel because of the inadequate sample size used and possible variability confounding the interpretation (Lee *et al.*, 1983).

6.2 Chromosome Aberration Studies

Several studies are available for the induction of chromosome aberrations in mammal systems using both *in vitro* and *in vivo* assays. Although the *in vitro* results have been conflicting, the majority of available *in vivo* data indicate that atrazine is not clastogenic (chromosome breaking), particularly in the bone marrow or in germ cells of the mouse. The few positive findings found *in vitro* tests are likely the result of cellular toxicity or stress and not a direct DNA mechanism of action. It should be noted that *in vitro* cytogenetic assays tend to have a relatively high frequency of sporadic positive responses that are usually associated with toxicity or other nonmutagenic events (*e.g*., high osmolality, low pH) (Brusick *et al.*, 1998).

6.2.1 *In Vitro* Assays

Atrazine did not produce chromosomal aberrations in Chinese hamster cells (Ishidate, 1988). A marginal increase in chromosome aberrations (less than a doubling in the response over background, and may be within the variation of background) was reported in human peripheral blood lymphocytes up to 1.0 μg/mL (Meisner *et al.,* 1992; Meisner *et al.*, 1993). By contrast, Lioi *et al.,* 1998, reported a large increase in the incidence of chromosome aberrations in human blood peripheral blood lymphocytes was reported at a similar dose (Lioi *et al*., 1998). It should be noted that agents that are mutagenic/clastogenic generally induce sister chromatid exchanges (SCE's) at lower doses. Thus, in the study by Lioi *et al.* (1998), it is unusual that such a strong dose-related response for chromosome aberrations was accompanied by only a marginal increase in SCE's (which was not dose-related) over the same concentration range (5 to 51 μM). Other studies using human peripheral blood lymphocytes, found atrazine to be negative up to a concentration of 10 μg/mL (Dunkelberg *et al.*, 1994) or up to 50 μg/mL (Kligerman *et al.*, 2000a) for SCE induction.

The lack of a clear SCE response suggests that the Lioi *et al.* positive findings for chromosome aberrations may reflect cellular toxicity or stress. Lioi *et al.* state that their positive chromosomal aberration response for atrazine "...indicated an induction of a pro-oxidant state of the cells as an initial response to pesticide exposure." given the increase found for glucose 6-phosphate dehydrogenase activity in exposed cells. Positive results were reported using flow cytometry methods in Chinese hamster ovary cells (Rayburn and Biradar, 1995.) This study is flawed and considered inconclusive because the method of cell lysis and staining of the nuclei used by the authors may have introduced artifacts. Flow cytometry analysis (which essentially measures the distribution of DNA between cells undergoing mitosis) is not as reliable as direct cell analysis by microscopy for evaluating clastogenicity. Many factors can alter the flow cytometry results, such as cleanliness of the machine, flow rate, cell number, air bubbles, incomplete cytolysis, incomplete RNA'ase digestion and sample preparation, thus making it different to interpret induced genetic changes by a chemical versus induced artifacts due to study conduct.

EPA NHEERL conducted an *in vitro* cytogenetic study on atrazine (as well as simazine and cyanazine) to resolve the contradictory cytogenetic findings reported in the literature on human peripheral blood lymphocytes. No induction of chromosomal aberration or SCE's was found in human peripheral blood lymphocytes after exposure to atrazine up to a dose of 50 μg/mL (Kligerman, *et al.*, 2000a).

6.2.2 *In Vivo* Assays

Atrazine administered in drinking water at 20 μg/mL (20 ppm), was found to be negative in a 30- and 90-day mouse ($B6C3F_1$ males and females) study using metaphase analysis to evaluate the incidence of chromosomal aberrations (Meisner *et al.*, 1992; Roloff *et al.*, 1992). It should be noted that bone marrow cytogenetic evaluations with out chromosome painting are insensitive for chronic studies. Atrazine tested negative in a mouse (Tif:MAGf) bone marrow assay evaluating micronuclei induction (Ceresa, 1988a). The study by Ceresa (1988a) consisted of two parts. In the first phase, both sexes of mice were dosed with a single gastric intubation of 2250 mg/kg atrazine in carboxymethyl cellulose, with animal sacrifices at 16, 24 or 48 hours following treatment. In the second phase of the study, both sexes of mice were treated with a single dose of atrazine at 562.5, 1175 or 2250 mg/kg with bone marrow cells harvested 24 hours post-treatment.

More recently, Gebel *et al.* (1997) examined a variety of herbicides, including atrazine in the mouse bone marrow micronuclei assay. In this study, NMRI mice of both sexes were gavaged with several doses of atrazine dissolved in corn oil up to 1750 mg/kg, and 48 hours later the animals were sacrificed. The results from this study showed that atrazine only induced a small increased in micronuclei in female mice at a dose of 1400 mg/kg that is approximately 80% of the LD_{50}. It should be noted that this is a very high dose of atrazine because at the next higher dose (1750 mg/kg), half the animals died (*i.e.*, it was the LD_{50}). The study authors state that *"(A)trazine... revealed significant aneugenic/clastogenic activities in the micronucleus test in vivo in female NMRI mice. However, these results only could be achieved in female animals at doses near to the maximum tolerated dose. Thus, an in vivo genotoxic potential for ... Atrazine seems questionable."* Although Adler (1980) cites a mouse bone marrow cytogenetic study in which atrazine was given by oral gavage and was found to be positive for clastogenetic effects at 2000 mg/kg (also a very high dose), no further details were provided to evaluate the acceptability of this finding. It should be noted that EPA's NHEERL found both atrazine and simazine to be negative for micronuclei induction in mice (Kligerman, *et al.*, 2000b).

Atrazine has been evaluated in dominant lethal assays for germ cell chromosomal damage. In a negative study by Hertner (1993), male mice were exposed via oral gavage up to 2400 mg/kg bw of atrazine. Males were mated sequentially with untreated virgin females at different days to allow evaluation of exposed male gametes at various germ cell stages of development. There were no significant increases for resorptions or dead fetuses at any dose. Although Adler (1980) cites a dominant lethal study in the mouse in which atrazine given by oral gavage caused an increase in dominant lethal mutations at 1500 and 2000 mg/kg, the lack of details precludes an independent assessment of the study.

6.3 Other Indicators of DNA Damage or Mutagen Exposure

Atrazine has been negative for the induction of unscheduled DNA synthesis (UDS) in rat hepatocyte cultures (Hertner, 1992; Puri and Muller, 1984). In the study by Hertner (1992), there was no evidence of UDS when hepatocytes from adult male Tif:RAIf rats were exposed *in vitro* to atrazine at several concentrations up to 1670 μg/mL for 16 to 18 hours (139 μg/mL was a precipitating concentration). In agreement, atrazine exposure did not induce UDS in the study by Puri and Muller (1984), which also used primary rat hepatocytes from adult male Tif:RAIf rats that were exposed to several concentrations of atrazine for five hours up to 150 µg/mL, where precipitation of the test article occurred.

Ribas *et al.* (1995) used the single cell-gel electrophoresis assay (SCGE, or the comet assay) to examine DNA strand breakage in human lymphocytes treated *in vitro* with several concentrations up to 200 µg/mL of atrazine for four hours both with and without S9 rat liver activation. In this study, atrazine was found to cause a marginal increase in alkaline labile sites only in the absence of mammalian liver S9 activation. The study authors refer to the atrazine results as a “weak positive” and noted: “The extent of DNA migration showed that only in cultures treated without S9 fraction there was a slight but significant increase and this took place only when the concentrations were high (100 and 200 µg/mL).” The weak positive findings by Ribas *et al.* were similar to the weak effect reported by another laboratory using the DNA alkaline elution assay to detect DNA strand breakage in stomach, kidney, and liver of Sprague-Dawley female rats treated with a single oral dose of 875 mg/kg of atrazine or with 350 mg/kg of atrazine given five or 15 successive days (Pino *et al.*, 1988). Another study using an excision repair assay to evaluate atrazine for DNA damage in

human lymphocytes up to a dose of 100 µg/mL, reported negative results without activation (Surrallés *et al.,* 1995). Although a positive finding was reported in another comet assay evaluating DNA damage in the tadpole, *Rana catesbeiana* (Clements *et al.,* 1997), it is uncertain whether this finding can be attributed to atrazine because the study was conducted with commercial formulation (Aatrex) that was of low atrazine purity (43%). It should be noted that Roundup was reported as positive in this study. The active ingredient in Roundup, glyphosate, is nonmutagenic when tested in standard genotoxicity assays (Flowers and Kier, 1978; Li, 1983; Shirasu *et al.,* 1978). Atrazine was also found to negative in an SOS chromotest (Ruiz and Marzin, 1997).

6.4 Mutagenicity Studies in Plants

Plant assays have yielded mix results. The induction of mutations but not chromosome aberrations have been observed in *Zea mays* (Morgun *et al.,* 1982). Conflicting results are reported in *Hordeum vulgare* for both mutation and chromosome aberrations (Wuu and Grant, 1966; Stroev, 1968; Muller *et al.,* 1972). The induction of chromosome aberrations is found when a high enough dose is evaluated in *Vicia faba* (Wuu and Grant, 1967). Although the metabolism in plants is qualitatively similar to that in mammalian, quantitative differences may exist in certain plant systems that may allow for expression of mutagenicity.

6.5 Metabolites of Atrazine

Metabolism of the triazine herbicides, atrazine, simazine and propazine, in mammalian species results primarily in chloro-s-triazine metabolites (Simoneux, 1995). The major pathway for metabolism of the triazine herbicides in plants is hydroxylation (Simoneux, 1995). The major plant metabolite is hydroxy-atrazine. Several standard mutagenicity studies on hydroxy-atrazine and a variety of these chloro-metabolites (Diaminochlortriazine, G-28279, and G-30033) have been consistently negative for mutagenicity, and thus do not appear to exhibit a mutagenic potential (summarized in Appendix Table 4).

Though not truly a metabolite of atrazine, the mutagenic potential of *N*-nitrosoatrazine is also discussed.

6.5.1 Diaminochlortriazine metabolite (DACT – 6-chloro-1,3,5-

triazine-2,4-diamine; didealkyl atrazine)

DACT (the structure is shown in Figure 8-1) was negative in the *Salmonella*/Ames assay when evaluated up to the limit concentration of 5000 µg per plate in tester strains TA 98, TA100, TA1535, and TA1537 with and without metabolic activation from the S9 fraction of Aroclor-treated rats (Deparde and Karimi, 1987). In an UDS assay using isolated human fibroblasts, DACT was also negative up to 600 µg/mL (which exceeded the solubility limit of 400 µg/mL) (Meyer, 1987).

6.5.2 G-28279 metabolite - 6-chloro-N-ethyl-1,3,5-triazine-2,4-diamine; deisopropyl atrazine

G-28279 (structure is shown in Figure 8-1) was tested up to a concentration 5000 µg per plate and found to be negative in the *Salmonella*/Ames assay when evaluated in tester strains TA 98, TA100, TA1535, and TA1537 with and without metabolic activation from the S9 fraction of Aroclor-treated rats (Deparde, 1990). G-28279 was also negative for inducing UDS in exposed hepatocytes from adult male Tif:RAIf rats when tested up to a cytotoxic dose (800 µg/mL) (Gelnick, 1991a). G-28279 was tested at the maximum tolerated dose of 480 mg/kg without inducing an increase in micronuclei in bone marrow cells of exposed adult Tif:MAGf mice of both sexes (Ogorek, 1991a).

6.5.3 G-30033 metabolite - 6-chloro-N-(1-Methyl ethyl)-1,3,5-triazine-2,4-diamine; deethyl atrazine

G-30033 (structure is shown in Figure 8-1) was tested up to a concentration 5000 µg per plate and found to be negative in the *Salmonella*/Ames assay when evaluated in tester strains TA 98, TA100, TA1535, and TA1537 with and without metabolic activation from the S9 fraction of Aroclor-treated rats (Deparde, 1989). G-30033 was also negative for inducing UDS in exposed hepatocytes from adult male Tif:RAIf rats when tested up to a cytotoxic dose (1000 μg/mL) (Gelnick, 1991b). G-30033 was tested at the maximum tolerated dose of 480 mg/kg without inducing an increase in micronuclei in bone marrow cells of exposed adult Tif:MAGf mice of both sexes (Ogorek, 1991b).

6.5.4 Hydroxy-atrazine, G-34048

Hydroxy-atrazine (structure is shown in Figure 8-1) was negative when evaluated at concentrations up to 5000 μg/plate in Salmonella tester strains TA 98, TA100, TA1535 and TA1537 (Deparde, 1988). Tests were conducted in the presence and absence of mammalian metabolic activation S9 fraction of Tif:RAIf rats treated with Aroclor 1254. When hepatocytes from adult male Tif:RAIf rats were exposed to hydroxy-atrazine at concentrations up to 1500 µg/mL (precipitation seen at doses $\geq$12.5 µg/mL), no increase in UDS was found (Hertner, 1988). Hydroxy-atrazine was negative in a UDS assay in which human fibroblast cells were exposed *in vitro* to concentrations up to 1500 µg/mL under nonactivating conditions only (Meyer, 1988). *In vivo*, hydroxy-atrazine was tested up to the limit dose of 5000 mg/kg without inducing an increase in micronuclei in bone marrow cells of exposed adult Tif:MAGf mice (Ceresa, 1988c).

6.5.5 *N*-Nitrosoatrazine

N-Nitrosoatrazine (NNAT) can be formed *in vitro* when atrazine and nitrite are mixed at an acid pH (Wolfe, *et al.*, 1976). Because nitrites and atrazine can be found together in drinking water, the hypothesis has been advanced that NNAT can be formed in the acid pH found in the stomach. The formation of NNAT in the stomach *in vivo* has yet to be demonstrated.

The genotoxicity of NNAT has been tested in the Ames assay, V-79 mutation assay, newt micronucleus test, and a clastogenicity assay using human lymphocytes.

Results of a modified Ames assay (available only as an abstract) showed NNAT to cause an increase in revertants in the TA 100 and 98 strains with hamster S9 fraction at 525 µg/plate and 1 mg/plate, respectively (Weisenberger *et al.*, 1987). The study authors considered these results to be "mildly mutagenic." This abstract also noted that atrazine was tested and found to be nonmutagenic.

A V79 assay (available only as an abstract) was considered by the study investigators to produce results that indicated that NNAT was "strongly mutagenic" (Weisenberger *et al.*, 1988). This abstract also noted that atrazine was tested and found to be nonmutagenic.

A micronucleus assay using peripheral red blood cells (RBC) from newt larvae resulted in an increase micronuclei at doses of 7.5 and 15 ppm after a 12 day exposure while no increase in micronuclei was seen at 3.75 ppm (Haridon, 1993).

A clastogenicity assay in lymphocytes from normal human volunteers found that NNAT at doses as low as 0.0001 µg/mL produced significant elevations of chromosome break frequency and percent of cells with aberrations. A significant elevation of the mitotic index was not observed at 0.0001 µg/mL. Mitotic index was significantly increased at the next highest dose of 0.001 µg/mL (Meisner, *et al.*, 1993).

6.6 Close Structural Analogues: *Simazine and Propazine*

As discussed in Chapter 8, atrazine is an s-triazine pesticide and is closely related to simazine and propazine as 2-chloro-4,6-bis-(alkyamino)-s-triazines. To further explore the mutagenicity of atrazine, the available databases on propazine and simazine were also evaluated. The available studies are predominantly negative, and thus do not provide convincing evidence of a mutagenic potential for simazine or propazine. Although there were some positives reported they tended to be marginal, found at very high concentrations or were not reproducible.

6.6.1 Simazine

As summarized is in Appendix Table 6 simazine has been evaluated in bacterial for mutation including various *Salmonella* tester strains and found to be negative by several different laboratories even when metabolic activation was incorporated into the assay (Mersch-Sundermann *et al.*, 1988; Simmon *et al.*, 1977; Jones *et al.*, 1984; Seiler, 1973; Lasinski, Kapeghian, and Green, 1987; Fahrig, 1974). Simazine was negative for the induction of gene mutation and gene conversion in *S. cerevisiae* (Fahrig, 1974; Siebert and Lemperle, 1974; Simmon *et al.*, 1977; Jones *et al.*, 1984; Emnova *et al.*, 1987). Like atrazine, conflicting results were reported in plant and insect studies. Only one mammalian *in vitro* gene mutation assay was found for simazine that reported a weak positive in the presence of metabolic activation (Jones *et al.*, 1984). This study is considered inconclusive by an expert Gene-Tox panel (Mitchell *et al.*, 1997). Simazine is also negative at concentrations up to the solubility limit in the primary rat hepatocyte UDS assay (Hertner, 1992).

Most *in vitro* cytogenetic assays on simazine are inconclusive due to study design problems, but negative results have been reported for the induction of SCE's and chromosomal aberrations. To resolve the inconclusive cytogenetic literature, EPA NHEERL recently evaluated simazine in human peripheral blood lymphocytes with *in vitro* exposures up to 37.5 μg/mL (a dose at the limit of toxicity and solubility) and found no induction of either chromosomal aberrations or SCE's (Kligerman *et al.*, 2000b). *In vivo*, simazine was tested up to the limit dose of 5000 mg/kg without inducing an increase in micronuclei in the bone marrow of exposed adult Tif:MAGf mice of both sexes (Ceresa, 1988b). EPA's NHEERL found simazine to be negative when evaluated by the mouse micronucleus test (Kligerman, *et al.*, 2000b).

6.6.2 Propazine

The available genotoxicity studies on propazine are summarized in Appendix Table 7. Propazine has been negative in bacterial mutagenicity assays (Kappas, 1988; Shirasu, 1975). Propazine has also been evaluated *in vitro* for the induction of gene mutation (at the HPRT locus) in Chinese hamster lung cells (V79) under both activating and nonactivating conditions (Ciba-Geigy, 1986). Studies without metabolic activation exposed V79 cells for 21 hours up to 1000 µg/mL of atrazine. Studies with metabolic activation exposed V79 cells for five hours up to 2000 µg/mL of atrazine along with the S9 fraction from Arochlor-treated male rats. In the experiment without S9 activation, a weak positive response (dose-related) was found at concentrations 400 µg/l. An equivocal response that was not dose-related was seen in the experiment with S9 activation.

Propazine was negative for UDS when tested up to the solubility limit in (62.5 µg/mL) exposed hepatocytes from adult, male TiF:RAIf rats (Puri, 1984). Propazine was reported to be negative for chromosome aberrations in Chinese hamster cells *in vitro* (Ishidate, *et al*., 1981; Ishidate 1983),and negative for the induction of micronuclei when tested up to 5000 mg/kg in adult female Chinese hamsters (Ciba-Geigy, 1984).

6.7 Summary and Discussion of Mutagenicity Data

The genetic toxicology database for atrazine shows consistent negative results for bacterial mutation assays. Beyond the bacterial tests, the database is heterogeneous and contains conflicting test responses. Reported positive responses tended to be weak and found at high doses of atrazine. No subset of data points clearly establishes a direct DNA reactive mode of action for atrazine associated with the carcinogenicity. Although the DNA damaging potential of atrazine can not be entirely dismissed in Drosophila and plants, these finding may be the result of species specific metabolism. Although some positive findings were reported for clastogenicity in mammalian systems, these responses were in conflict with other studies using the same assay approach and may be associated with toxicity. Data on several metabolites of atrazine and its close structural analogues (propazine and simazine) do not support a mutagenic potential for these chemicals.

In summary, the totality of evidence does not support a mutagenic potential for atrazine, and indicates that a direct DNA reactive/mutagenic mode of action is unlikely to be a component of atrazine-induced mammary gland neoplasia (or on tumor development at any other site).

N-nitrosoatrazine has been shown to be mutagenic in four different types of mutagenicity assays. However, the chemical reaction which generates NNAT has never been demonstrated to occur *in vivo*, and cancer bioassays in female Swiss mice and female Wistar rats failed to show a carcinogenic response following NNAT exposure (Weisenberger,1990 - abstract).

In conclusion, exposure to atrazine is not likely to pose a mutagenic hazard to humans, especially at lower exposure levels experienced by humans.

Chapter 7

7 Estrogenic Activity

Several studies employing both *in vitro* and *in vivo* assays are available concerning the potential of atrazine to act as an estrogen agonist *(i.e.*, mimic the effects of estradiol exposure). Some of the assays also test the ability of atrazine to function as a progesterone mimic. The results of these studies together provide evidence that atrazine does not have direct estrogenic effects. These studies are discussed below and their results are also summarized in Table 7-1.

7.1 *In Vivo* Assays

Tennant *et al.* conducted a study known as the uterotrophic response assay. This is an accepted procedure for measuring estrogenic activity (Korach and McLachlan, 1995). As an *in vivo* assay, it incorporates aspects such as metabolism, serum binding and pharmacokinetics. Exposure to an estrogenic agent increases the weight of the uterus by causing cellular proliferation of endometrial cells, leading to an increase in thickness of the uterine endometrium. Rats or mice are exposed to the suspected estrogenic agent for three or four days, sacrificed, and their uterine weight is compared to that of the control animals. Immature or OVX animals are used to decrease interference from endogenous estrogens.

In this study, OVX Sprague-Dawley rats were treated for three days with 20, 200 or 300 mg/kg of atrazine. An increase in uterine weights over controls was not found.

Tennant *et al.* (1994a) also evaluated atrazine for uterine thymidine incorporation. This test measures an increase in uterine cell proliferation. Rather than measuring proliferation of uterine cells by weighing the uterus, this test measures the incorporation of thymidine into cellular DNA prior to cell division.

In this assay, female Sprague-Dawley rats were fed radiolabeled thymidine in their diet and then exposed to 1, 10, 50, 100 or 300 mg/kg/day atrazine for three days. The atrazine-treated animals had less thymidine incorporation into uterine cells than did control rats.

Table 7-1. *In vitro* and *in vivo* Hormonal Studies with Atrazine

Study Type	Dose/Duration	Results
In vivo Uterine bioassay in OVX SD females[1]	Atrazine and DACT at 0, 20, 200 or 300 mg/kg for three days. Simazine at 100 and 300 mg/kg/day for three days.	*An increase in uterine weight indicates estrogenic activity* **+ control** - estradiol at 2 µg stimulated uterine weight gain; **Atrazine**/DACT/Simazine - uterine weights in dose groups were similar to control weights.
In vivo Progesterone receptor binding assay in OVX SD females [1]	Atrazine at 0, 50 or 300 mg/kg for three days followed by 5 µg/kg E2	*An increase in the ability of progesterone receptor (PR) to bind its agonist indicates estrogenic activity* **+ control** - estradiol at 2 µg increased PR binding of agonist; **Atrazine** - 300 mg/kg/day decreased the ability of PR to bind a PR agonist; **DACT** - 300 mg/kg/day decreased the ability of PR to bind a PR agonist; **Simazine** - 300 mg/kg/day decreased the ability of PR to bind a PR agonist
In vitro Uterine Thymidine Incorporation assay[1]	Atrazine at 0, 1.0, 10, 50, 100 or 300 mg/kg for three days followed by 0.15 µg of E2	*An increase in uterine thymidine incorporation indicates estrogenic activity* **+ control** - estradiol at 0.15 µg increased uterine thymidine incorporation; **Atrazine** - 300 mg/kg/day decreased uterine thymidine incorporation; **DACT** - 300 mg/kg/day decreased uterine thymidine incorporation; **Simazine** - 300 mg/kg/day decreased uterine thymidine incorporation
In vitro MCF-7 Cell Proliferation Assay[2]	Atrazine/simazine at 0.01, 0.1, 1.0, 10 and 100 µM for 11 days	*An increase in the proliferation of MCF-7 cells indicates estrogenic activity* **+ control** - 1 nM estradiol induced a two-fold increase in cell number; **Atrazine/simazine** - no increase in cell numbers was seen at any dose of these compounds
In vitro Gel-shift assay for PR-PRE complex[2]	Atrazine/simazine at 1 µM	*An increase in the retardation through an electrophoretic gel of a complex consisting of PR + PR agonist + PR DNA binding region, indicates estrogenic activity* **+ control** - 1 nM estradiol resulted in a large increase in the retardation of the complex through a gel; **Atrazine/simazine** -Movement of the complex through the gel was not altered by these compounds

In vitro Luciferase reporter gene assays in MCF-7 cells[2]	Atrazine/simazine at 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} M	*An increase in luciferase activity indicates estrogenic activity* **+ control** - estradiol at doses as low as 10^{-12} M resulted in increases in luciferase activity **Atrazine/simazine** - no increase in luciferase activity was seen with either compound at any dose.
In vitro Estrogen receptor competitive binding assay using uteri from rats that *had not* previously been exposed to triazines[3]	Atrazine/simazine/DACT at 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3} for equilibrium conditions A variety of experiments were also run in which conditions favored triazine binding to the estrogen receptor. These were termed disequilibrium conditions.	*Displacement of estrogen from its receptor indicates estrogenic activity* No displacement of estradiol from its receptor was observed at any dose with any of the triazine compounds under equilibrium conditions Under conditions that favor triazine binding to the estrogen receptor (disequilibrium conditions) triazine will displace estrogen from its receptor.
In vitro Estrogen receptor competitive binding assay using uteri from rats *previously exposed* to triazines[3]	Atrazine/simazine/DACT at 50 and 300 mg/kg/day for two days to OVX SD females	*Displacement of estrogen from its receptor indicates estrogenic activity* At 50 mg/kg/day displacement of estrogen from it receptor was not significantly altered with any of the triazine compounds. At 300 mg/kg/day there was a significant decrease in estrogen binding with all three triazine compounds.
In vitro Yeast assay using transfected human estrogen receptor[4]	Both maximal (1.0x 10^{-9} M, in this system) and submaximal (2.5 x 10^{-9} M) concentrations of atrazine, and the chloratrazine metabolites alone and in varying combinations were tested	*An increase in â-galactosidase activity indicates binding of atrazine to the estrogen receptor* Nether atrazine alone, nor any of the many combinations of atrazine with the atrazine metabolites caused an increase in â-galactosidase activity.
In vitro Estrogen receptor mediated growth in yeast[5]	Atrazine and simazine at 10 µM	*Growth of the yeast indicates estrogenic activity* **+ control** - Yeast colonies exposed to estradiol at 1nM proliferated. **Atrazine/simazine** - Yeast colonies exposed to atrazine and simazine did not proliferate.
In vivo Uterine peroxidase assay using uteri from female SD rats	Atrazine and simazine at 50, 150, and 300 mg/kg/day were exposed female SD rat through gavage for three days	*Uterine peroxidase activity indicates estrogenicity* **+ control**- estradiol at 10 µg/day results in a 10-fold increase in uterine peroxidase activity. **Atrazine/simazine** - neither compound at any dose increased uterine peroxidase activity.

[1]Tennant *et al.*, 1994a.; [2]Safe *et al.*, 1995.; [3]Tennat *et al.*, 1994b.; [4]Graumann *et al.*, 1999; [5]Conner *et al.*, 1996.

7.2 *In Vitro* Assays

Safe *et al.* (1995) used MCF-7 cells, a human breast cancer cell line that proliferates best in the presence of estrogen, to evaluate the potential estrogenic activity of atrazine. In this method, cells are grown in culture medium that has been charcoal-filtered to ensure that it is free of estrogens. The assay is conducted by adding the suspected estrogenic compound to the cell's medium and measuring cell proliferation, usually by counting the cells.

MCF-7 cells exposed to 10 µM atrazine did not show increased proliferation over control cells in this study.

Tennant *et al.* (1994b) evaluated the ability of atrazine, simazine and diaminochlorotriazine (DACT, a metabolite of both atrazine and simazine) to bind to the rat uterine estrogen receptor. A uterine cytosol extract was prepared from female Sprague-Dawley rats. These preparations are expected to be rich in estrogen receptors. The uterine cytosol was incubated with radiolabeled estrogen and one of the test chemicals. After an appropriate incubation time, estrogen bound to its receptor was separated from unbound estrogen. High levels of unbound estrogen would indicate that one of the test compounds was competing with estrogen for binding to estrogen receptors.

Neither atrazine, simazine, nor DACT treatment was able to compete with estrogen for binding to the estrogen receptor. No competitive binding was apparent under conditions of equilibrium. Only when excessive amounts of triazines were used (10,000-fold molar excess), was a slight competitive binding observed. Atrazine, simazine and DACT were not considered, under the conditions of this study, to effectively compete with estrogen for binding to the estrogen receptor.

Tennant *et al.* (1994b) also used an assay approach similar to the one described above with the exception that the triazines were administered by oral gavage, and uterine slices were incubated with radiolabeled estrogen instead of cytosol extracts. The advantage of this method compared to the test without *in vivo* preincubation is that exposure to the triazine compounds is done *in vivo*.

In this study OVX female Sprague-Dawley rats were exposed to 50 or 300 mg/kg/day of either atrazine, simazine, or DACT for two days. The animals were sacrificed, uterine slices were prepared, and incubated with radiolabeled estrogen. Dosing at 300 mg/kg/day statistically reduced estrogen binding by 33% with atrazine, 39% with simazine and 24% with DACT. Dosing at 50 mg/kg/day reduced estrogen binding by 18% with atrazine, 21% with simazine and 13% with DACT, but values were not statistically-significant. At high doses, triazine compounds are able to bind to rat uterine estrogen receptors *in vivo*.

Conner *et al.* (1996) used the reporter gene assays to evaluate atrazine. In these experiments two constructs---a Gal4-HEGO chimeric receptor and a GAL4-regulated promoter containing five ERE's and the luciferase gene--were placed into the MCF-7 cell line. The luciferase gene is a reporter gene whose product can be easily measured because it emits light when cells are exposed to a compound that binds the estrogen receptor.

Treatment of these MCF-7 cells with estrogen in the nM range produced large increases in luciferase activity compared to controls. However, treatment with atrazine or simazine at doses as high as 10 µM failed to produce any increases in luciferase activity compared to controls. These experiments show that, in this system, atrazine and simazine fail to bind to the estrogen receptor.

Conner *et al.* (1996) evaluated atrazine estrogen-dependent growth yeast strain. The PL3 yeast strain requires for growth a medium supplemented with the amino acids histidine and leucine, and the pyrimidine base uracil. If these yeast cells were transformed so as to contain the human estrogen receptor, then estrogen could take the place of uracil and allow growth of this strain on histidine and leucine-containing medium. ER-positive PL3 cells in media supplemented with histidine, leucine and estrogen will show growth after only one day of culture and continue to proliferate for at least five days.

ER-positive PL3 cells supplemented with histidine, leucine and either 10 µM atrazine or 10 µM simazine show no proliferation even after five days of culture. This study demonstrates that, in a yeast cell expressing human ER, neither atrazine nor simazine have estrogenic effects.

Bradlow *et al.* (1995) and Safe and McDougal (1998) examined the 16á to 2-hydroxyestrone metabolite ratios after atrazine treatment. Estradiol forms many metabolites. It has been hypothesized by some investigators that the ratio of the 16 metabolites to the 2-hydroxyestrone (2-OHE) metabolites may be a factor in mammary carcinogenesis. It is believed that a high 16á to 2-OHE ratio may be correlated with mammary carcinogenesis (Telang *et al.*, 1997). Nevertheless, others question whether this ratio is truly predictive.

Bradlow *et al.* (1995), exposed MCF-7 cells to several different chemicals including atrazine. MCF-7 cells were placed in medium containing [16á-^{3}H] estradiol (to determine 16á metabolite levels) and [2-^{3}H] estradiol (to determine 2-OHE metabolite levels) and atrazine at 1×10^{-5} M. Incubation was for 48 hours following which measurements of the various metabolites were made. The 16á to 2-OHE ratio in the atrazine exposed cells was approximately seven to nine times higher than the negative control and three to four times higher than ratios seen with the known mammary carcinogen dimethyl benzanthracene (DMBA).

Safe and McDougal (1998) exposed MCF-7 cells to several different chemicals including atrazine. Exposure to atrazine was at 1×10^{-5} M for 48 hours followed by another 48 hours of atrazine plus [16á-^{3}H]estradiol or 48 hours of atrazine plus radiolabeled [2-^{3}H]estradiol. The ratio of 16ámetabolites to 2-OHE metabolites was not increased following exposure to atrazine (in fact it was somewhat decreased compared to negative controls).

The results with atrazine in this assay have been contradictory; one group of investigators (Bradlow *et al.,* 1995) reported an increased ratio, supportive of potential carcinogenicity, while another group (Safe and McDougal, 1998) could not confirm these results.

Tran *et al.* (1996) used a yeast strain transfected with the human estrogen receptor to evaluate atrazine. A yeast cell line was transfected with the human estrogen receptor linked to the *lacZ* gene. This system is similar to that used by Conner *et al.* described above; the major exceptions were that the cells are yeast rather than MCF-7, and the reporter system is â-galactosidase rather than luciferase. To examine the role of different domains of the estrogen receptor, these investigators also transfected a gene encoding for the human estrogen receptor minus the first 179 amino acids.

Atrazine, cyanazine, simazine and the DACT metabolite were used in concentrations ranging from 207 nM to 2075 nM for atrazine, simazine and DACT, and up to 10,000 nM for cyanazine. All exposures included estradiol at either 20 or 0.5 nM (referred to the by the study authors as maximal and sub-maximal concentrations, respectively). With both the full and the truncated receptor, no concentration of triazine, in combination with either 0.5 or 20 nM or estradiol, resulted in â-galactosidase activity greater than what was seen with estradiol alone.

The experiments done with the complete estrogen receptor plus 0.5 nM estradiol (but not 20 nM) showed that all four triazines resulted in less â-galactosidase activity than with estradiol alone. Studies with the truncated receptor showed that at all triazine doses with both 0.5 and 20 nM estradiol, â-galactosidase activity was not altered compared to estradiol alone. Under none of the conditions tested did any of the triazines have estrogenic activity. Anti-estrogenic activity was seen, but only with the submaximal concentration and not the maximal concentration of estradiol. Since, the anti-estrogenic activity was not seen in the experiments done with the truncated receptor, it can be assumed that the amino-terminus of the estrogen receptor is responsible for the anti-estrogenic activity.

Experiments by Graumann *et al.* (1999) used also employed yeast that had been transfected with DNA coding for the human estrogen receptor linked to a â-galactosidase expression system. Only the full receptor was used.

Yeast were exposed to atrazine or atrazine plus the atrazine metabolites Desethyatrazine (DE; 6-chloro-N-ethyl-1,3,5-triazine-2,4-diamine) and Desisopropylatrazine (DI; 6-chloro-N-(1-Methyl ethyl)-1,3,5-triazine-2,4-diamine) over a variety of concentrations and combinations. Atrazine alone, atrazine plus DE, atrazine plus DI and all three together were used. Like the Tran *et al.* study, both maximal (1.0×10^{-9} M, in this system) and submaximal (2.5×10^{-9} M) estradiol concentrations, along with varying concentrations and combination of triazines, were used.

In agreement with the data of Tran *et al.* (1996), no concentration or combination of triazines at either maximal or submaximal conditions resulted in an increase in â-galactosidase activity. In contrast to the Tran paper, no concentration or combination of triazines at either maximal or submaximal conditions resulted in a decrease in â-galactosidase activity either.

The Tran and Graumann papers, using similar systems, found that none of the triazine compounds tested possessed intrinsic estrogenic activity. The results in regards to anti-estrogenic activity are contradictory with one study (Tran) finding anti-estrogenic activity and one study (Graumann *et al.*,1999) not finding such activity.

A study has been published which examines the upregulation of aromatase activity by atrazine. Aromatase - also known as cytochrome P450 19 (CYP19) - is the enzyme responsible for the conversion of androgens to estrogens (specifically, the catalysis of androstenedione to estrone and testostrone to estradiol). An upregulation of CYP19 activity would be expected to result in increased serum estrogen levels as an increased amount of androgens are converted to estrogens. An increase in CYP19 activity has been observed following atrazine treatment in a human adrenocortical carcinoma cell line - H295R (Sanderson *et al.*, 2000).

7.3 Special Carcinogenicity Bioassay Study (Morseth, 1998)

As discussed in Chapter 5, in a two-year oncogenicity study (Morseth, 1998), groups of estradiol-implanted OVX and intact female SD rats were exposed to 97.1% atrazine at doses of 0, 25, 50, 70 and 400 ppm. The primary purpose of this study was to examine the effect of ovariectomy on mammary tumor development in the SD rat following atrazine exposure. This study also provides information concerning the potential estrogenic activity of atrazine in relation to mammary carcinogenesis.

If atrazine induces mammary tumors by acting directly on mammary epithelium, then OVX animals might be expected to develop mammary tumors as do intact animals. Removal of the ovaries of an animal should not have affected the ability of atrazine to affect mammary epithelial cells and commence carcinogenesis. However, the difference in mammary tumor incidence between OVX and intact animals was striking. Not a single OVX animal in any dose group developed a mammary tumor of any type. In contrast, among intact animals, 38.3% of controls and 68.3% of the 400 ppm group developed mammary neoplasia.

Overall, the results from this study, showing a complete lack of mammary tumors in OVX animals, provides further evidence that atrazine does not induce mammary tumors by binding to and activating mammary tissue estrogen receptors. This evidence is also consistent with the conclusion that mutagenicity is not a key component of atrazine's carcinogenicity.

7.4 Noncancer Effects Relevant to Estrogenic Activity

A variety of bioassays using atrazine have been submitted to the Agency. Many of these assays measure parameters that might be expected to be altered were atrazine acting as an estrogenic agent. This section examines subchronic dog and rat studies; chronic dog studies; multi-generation reproduction studies in the rat; and developmental toxicity studies in the rat and rabbit, for alterations in parameters that may be indicative of a possible estrogenic effect.

Alterations in the chronic dog and subchronic rat and dog studies that were considered to possibly indicate an estrogenic effect were: changes in testes or prostate weights in males; changes in ovarian or uterine weight in females; histopathology findings in the testes (including seminiferous tubules) or prostate of males; histopathology findings in the ovaries, uterus, vagina or mammary gland of females.

Data in the multigeneration studies that were examined included: parental testes, ovary and uterine weights; parental histopathology findings in the testes or prostate of males; histopathology findings in the ovaries, uterus, vagina or mammary gland of female parents; malformations or variations of the testes and prostate of male offspring and the ovaries, uterus, vagina or mammary gland of female offspring; and, the ability of male offspring to impregnate females and the ability of females to become pregnant and deliver healthy pups.

In the developmental toxicity studies, data from visceral examinations were examined for malformations or variations of the gonads in offspring. In addition, the ratio of male to female offspring was examined. Maternal organ weights and histopathology were not determined in these studies (which were conducted under the Subdivision F guidelines). Thus, maternal organ weight and histopathology data are not available to analyze for potential estrogenic effects.

In addition to examining data from the above mentioned atrazine bioassays, data from bioassays with simazine and the atrazine/simazine metabolites DACT, G-28279, and G-30033 were also scrutinized for potential estrogenic effects.

7.4.1 Subchronic Dog Studies

Subchronic studies with atrazine and propazine in the dog are not available, but subchronic dog studies using simazine, G-28279 and G-30033 are available. Prostate and uterine weights were determined in the G-28279 and G-30033 studies, but not in the simazine or DACT studies.

Simazine. Testes weights in male Beagle dogs were decreased (46% decrease in absolute testes weight and 27% decrease in testes weight relative to body weight, compared to controls) in male Beagle dogs exposed to the high dose of 133.6 mg/kg/day of simazine. Ovary weights in females were not significantly altered at any dose including the high dose tested of 136 mg/kg/day. No increases in histopathology findings in either the male testes or prostate or the female ovaries, uterus, vagina or mammary gland were observed in dose groups compared to controls (Tai *et al.,* 1985a)

G-28279. Testes weights in male Beagle dogs exposed to the two highest doses tested in this study of 18.9 or 33.4 mg/kg/day were decreased. Compared to controls absolute testes weight decreased 31.4% at 33.4 mg/kg/day and testes weight relative to brain weight was decreased 36.7%. The decreases compared to controls in the 18.9 mg/kg/day group were 22.85 and 25.3% for absolute testes weight and testes weight relative to brain weight. Prostate weight in these two dose groups were also reduced compared to controls. The percent reductions at the high dose were 60.2% and 62.6% for absolute weight and prostate weight relative to brain weight. The reductions in the 18.9 mg/kg/day group were 51.55 and 51.9% for absolute weight and for prostate weight relative to brain weight. Ovary or uterus weights in the females of any dose group were not altered compared to controls. No increases in histopathology findings in either the male testes or prostate or the female ovaries, uterus, vagina or mammary gland were observed in dose groups compared to controls (Thompson *et al.*, 1992).

G-30033. Male testes and prostate weights were not significantly altered in Beagle dogs exposed to a high dose 28.85 mg/kg/day. Females ovary weights were not altered in females exposed to a high dose of 32.18 mg/kg/day. Both absolute uterine weights and uterine weight relative to brain weight were significantly decreased in the high-dose females. However, these changes were deemed by the study reviewer to likely be secondary to body weight loss rather than a direct effect of G-30033 exposure. No increases in histopathology findings in either the male testes or prostate or the female ovaries, uterus, vagina or mammary gland were observed in dose groups compared to controls (Rudzki *et al.*, 1992).

DACT. Male and female Beagle dogs used in this study showed no significant alteration in testes or ovary weight up to the high doses of 24.1 and 32.7 mg/kg/day for males and females respectively. There was no increase in histopathology findings in the female ovary, uterus, vagina or mammary gland in dose groups compared to controls. There was, however, an increased incidence of hypospermia and hypospermatogenesis in all four of the 24.1 mg/kg/day males, while no animal in any of the other group, including the controls, displayed these findings (Thompson *et al.*, 1990).

7.4.2 Subchronic Rat Studies

Subchronic studies with atrazine and propazine in the rat are not available, but subchronic rat studies using simazine, G-28279 and G-30033 are available.

Simazine. Absolute testes weights were decreased (15.4%) in male SD rats exposed to the high dose of 276 mg/kg/day of atrazine while testes weights relative to body weight were not altered compared to controls. Absolute ovary weights in the females exposed to either 142 or 276 mg/kg/day were reduced by 20% and 40%, respectively, while relative ovary weights were not altered compared to controls. No increases in histopathology findings in either the males testes or prostate or the female ovaries, uterus, vagina or mammary gland were observed in any dose groups compared to controls (Tai *et al.*, 1985b).

G-28279. Male SD rats showed increased testes weights relative to body weight at the high dose tested (34.9 mg/kg/day) in this study. Relative testes weights were increased 139% compared to controls, but absolute testes weights at this dose were within 5% of control values. Ovary weights were not significantly altered at any dose. No increases in histopathology findings in either the testes or prostate or the ovaries, uterus, vagina or mammary gland were observed in any dose groups compared to controls (Schneider, 1992).

G-30033. Testes and ovary weights of the RAIf rats used in this study were not significantly altered even at the high does of 35.1 for males and 38 mg/kg/day for females. No increases in histopathology findings in either the males testes or prostate or the female ovaries, uterus, vagina or mammary gland were observed in dose groups compared to controls (Gerspach, 1991).

DACT. Sprague-Dawley rats were exposed to doses of DACT of up to 34.1 mg/kg/day for males and 40.2 mg/kg/day for females. Testes weights relative to body weight were increased 22% at 34.1 mg/kg/day and absolute testes weights were increased 6.6% compared to controls. No significant alterations in ovary weights were noted at any dose. No increases in histopathology findings in either the male testes or prostate or the female ovaries, uterus, vagina or mammary gland were observed (Pettersen *et al.*, 1991).

7.4.3 Chronic Dog Studies

Chronic (12-month) dog studies with atrazine DACT, and simazine are available. Chronic dog studies with propazine, G-28279 and G-30033 are not available.

Atrazine. The Beagle dogs used in this study showed no significant alteration in testes or ovary weights up to the high dose used of 33.65 and 33.8 mg/kg/day for males and females, respectively. There were no histopathology findings in the testes, prostate, ovaries, uterus, vagina or mammary gland (O'Connor *et al.*, 1987).

Simazine. Doses of up to approximately 43 mg/kg/day did not alter either absolute or relative testes or ovary weights in beagle dogs exposed to simazine for one year. No increases in histopathology findings in either the male testes or prostate or the female ovaries, uterus, vagina or mammary gland were observed in dose groups compared to controls.

DACT. The Beagle dogs used in this study showed no significant alteration in testes or ovary weights up to the high dose used of 24.1 and 32.7 mg/kg/day for males and females, respectively. There was no increased incidence of histopathology findings in the female ovary, uterus, vagina or mammary gland in dose groups compared to controls. There was an increased incidence of hypospermia and hypospermatogenesis in two of the four 24.1 mg/kg/day males, but not in any other group, including the controls (Thompson *et al.,* 1990).

7.4.4 Multi-Generation Reproduction Studies

Atrazine. Sprague-Dawley rats were exposed to atrazine at concentrations up to a high dose of 39 mg/kg/day for males and 42.8 mg/kg/day for females. Testes weight relative to body weight was significantly increased in both parental generations at the high dose (11.1% increase in the F_0 generation and 11.1% increase in the F_1 generation). Although slightly decreased compared to controls, absolute testes weights were within 10% of control values. Ovary weights were not significantly altered compared to controls. There was no increased incidence of histopathology findings in the testes, prostate, uterus, vagina or ovaries (histopathology was not done on the mammary gland) at any dose in either generation of parental animals, compared to controls. There were no necropsy or histopathology findings in the male offspring of atrazine-treated dams that would indicate excessive maternal exposure to estrogens (Mainiero *et al.,* 1987).

Simazine. Sprague-Dawley rats were exposed to simazine at concentrations up to a high dose of 28.89 mg/kg/day for males and 34.96 mg/kg/day for females. Testes weight relative to body weight was significantly increased in both parental generations at the high dose (11.5% increase in the F_0 generation and 20% increase in the F_1 generation). Absolute testes weights were within 10% of control values for both parental generations at this dose. Ovary weights relative to body weights were significantly increased in the F_1 parental generation only at the high dose. Absolute ovary weights in both parental generations at all doses, were not significantly altered compared to controls. There was not an increased incidence of histopathology findings in the testes, prostate, uterus, vagina or ovaries (histopathology was not done on the mammary gland) at any dose in either generation of parental animals, compared to controls. There were no necropsy or histopathology findings in the male offspring of atrazine-treated dams that would indicate excessive maternal exposure to estrogens (Epstein *et al.,* 1991).

Propazine. Sprague-Dawley rats were exposed to doses of up to 50 mg/kg/day of propazine in this three-generation study. F_0 paternal testes weights relative to body weight, at the high dose only, were significantly increased (15.6%) compared to controls. Absolute testes weights at this dose (and every other dose) in this generation were within 5% of control values. Testes weight relative to body weight in the F_1 generation was not significantly altered though it was increased 16.6% in the high-dose groups compared to controls. Absolute testes weight in all dose groups was within 5% of control values. Testes weights relative to body weights were significantly increased (22%) compared to controls in the high-dose of the F_2 generation parents. Absolute testes weights were within 5% of control values for all other dose groups tests in this generation. Ovary weights in the first two generations were not significantly different, either absolutely or relative to body weight, than control ovary weights. Ovary weights in the F_2 generation parents were significantly decreased (27.6%) at the mid-dose of
5 mg/kg/day. The ovary weights at the other two doses in this generation were higher than the ovary weights at 5 mg/kg/day and were within 10% of control values. There was not an increased

incidence of histopathology findings in the testes, prostate, uterus, vagina or ovaries (histopathology was not done on the mammary gland) at any dose in any generation of parental animals, compared to controls. There were no necropsy or histopathology findings in the male offspring of atrazine-treated dams that would indicate excessive maternal exposure to estrogens (Jessup, 1979).

7.4.5 Rat Developmental Toxicity Studies

Atrazine. Pregnant Sprague-Dawley rats were exposed to up to 100 mg/kg/day of atrazine from days six to 15 of their pregnancies. There were no findings, in the visceral examinations, of any malformations or variations in the gonads at any dose. The sex ratios in all the dose groups were similar to the control sex ratio (Ginkis, 1991).

Simazine. Pregnant Sprague-Dawley rats were exposed to up to 600 mg/kg/day of simazine from days six to 15 of their pregnancies. There were no findings, in the visceral examinations, of any malformations or variations in the gonads at any dose. The sex ratios in all the dose groups were similar to the control sex ratio (Mainiero *et al.*, 1986).

Propazine. Pregnant Sprague-Dawley rats were exposed to up to 600 mg/kg/day of propazine from days six to 15 of their pregnancies. There were no findings, in the visceral examinations, of any malformations or variations in the gonads at any dose. The sex ratios in all the dose groups were similar to the control sex ratio (Fritz, 1976).

DACT. Pregnant Sprague-Dawley rats were exposed to up to 150 mg/kg/day of DACT from days six to 15 of their pregnancies. There were no findings, in the visceral examinations, of any malformations or variations in the gonads at any dose. The sex ratios in all the dose groups were similar to the control sex ratio (Hummal *et al.*, 1989).

G-30033. Pregnant Tif:RAI rats were exposed to up to 100 mg/kg/day of G-30033 from days six to 15 of their pregnancies. There were no findings, in the visceral examinations, of any malformations or variations in the gonads at any dose. The sex ratios in all the dose groups were similar to the control sex ratio (Gerspach, 1991).

G-28279. Pregnant Tif:RAI rats were exposed to up to 100 mg/kg/day of G-28279 from days six to 15 of their pregnancies. There were no findings, in the visceral examinations, of any malformations or variations in the gonads at any dose. The sex ratios in all the dose groups were similar to the control sex ratio (Marty, 1992).

7.4.6 Rabbit Developmental Toxicity Studies

Atrazine. Pregnant New Zealand White rabbits were exposed to up to 75 mg/kg/day of atrazine from days seven to 19 of their pregnancies. There were no findings, in the visceral examinations, of any malformations or variations in the gonads at any dose. The sex ratios in all the dose groups were similar to the control sex ratio (Arthur, 1984a).

Simazine. Pregnant New Zealand White rabbits were exposed to up to 200 mg/kg/day of simazine from days seven to 19 of their pregnancies. There were no findings, in the visceral examinations, of any malformations or variations in the gonads at any dose. The sex ratios in all the dose groups were similar to the control sex ratio (Arthur, 1984b).

Propazine. Pregnant New Zealand White rabbits were exposed to up to 50 mg/kg/day of atrazine from days seven to 19 of their pregnancies. One male offspring in the mid-dose group of 10 mg/kg/day was found to have absent testes. Otherwise, there were no findings, in the visceral examinations, of any malformations or variations in the gonads at any dose. The sex ratios in all the dose groups were similar to the control sex ratio (Knapp, 1995).

The bioassays described above provide little evidence of an estrogenic effect. Dosing in utero with estrogenic agents has been associated with findings such as cryptochidism, hypospadias and anorchism (Daston *et al.*, 1997; Danzo, 1998). Developmental toxicity studies in both the rat and rabbit and multigeneration reproduction studies in the rat, failed to show an increased incidence of these, or any other, abnormalities of the gonads. Multigeneration reproduction studies in the rat did not indicate that male offspring of atrazine-treated dams had any difficulties impregnating females although decreased fertility in males exposed to increased levels of estrogen or estrogen mimicking compounds in utero, has been described (Daston *et al.*, 1997; Danzo, 1998). Developmental toxicity studies in both the rat and rabbit and multigeneration reproduction studies in the rat, failed to show any alterations in sex ratios compared to controls. Ample evidence is available that estrogen exposure can result in germ cell depletion in the seminiferous tubules within a few weeks of commencement of estrogen administration (Blanco-Rodríguez and Martínez-García, 1996).

Subchronic rat and dog studies and chronic dog studies failed to show any histopathology alterations in the seminiferous tubules or in any part of the testes, or in the prostate. Excessive exposure to estrogen induces proliferation of the uterine endometrium. Subchronic rat and dog studies and chronic dog studies failed to show any histopathology alterations in the uterus, and, in the studies where uterine weights were determined, no alteration in uterine weights in response to triazine exposure were noted either. Excessive estrogen exposure might also be expected to result in alterations in the vagina - such as vaginal hyperplasia and vaginal wall thickening, or changes in the ovaries - such as an increase in ovarian stromal cells or enlarged ovaries. Subchronic rat and dog studies and chronic dog studies failed to show any histopathology alterations in the vagina or ovaries following exposure to the triazines. Ovarian weights were also not affected by triazine exposure.

The only potential endocrine alteration that is consistently seen in these bioassays is an alteration in either relative or absolute testes weight. This alteration is, however, somewhat variable. In seven rat studies and one dog study testes weights were increased; in two rat studies weight were decreased; in three dog studies and one rat study testes weights were not altered. Thus, in a total of 14 studies, testes weight was increased in eight studies, decreased in two, and not altered in four. Excessive estrogen stimulation would be expected to decrease testes weight.

The studies described above do not indicate an estrogenic effect of atrazine, simazine, propazine, DACT, G-30033, or G-28279. All the parameters examined for possible estrogenic effects were unaltered save one- testes weight. The testes weight alterations were variable, and the majority of the time they were altered, they were increased when one would expect that they would be decreased.

7.5 Overall Conclusions of Estrogenic Activity Data

Multiple studies that examine the estrogenic activity of atrazine, simazine and DACT have been reviewed by the Agency. Most, but not all, studies indicate that these chemicals do not possess estrogenic activity. Utertrophic response assays, uterine thymidine incorporation assays, MCF-7 cell proliferation assays, luciferase reporter gene assays in MCF-7 cells, *in vivo* progesterone receptor binding assays, uterine peroxidase assays, and estrogen receptor mediated growth in were all negative for estrogenic or progesterone activity of the triazine herbicides.

The few that yielded positive results were performed under conditions that favored an estrogenic effect of atrazine, or were performed at very high dose levels relative to the doses that induce mammary tumors in SD females.

A study examining atrazine's effects on aromatase activity indicated an upregulation of aromatase *in vitro* activity following atrazine treatment. The role this finding may play in bringing about an increase in serum estrogen *in vivo* is unclear as this study was conducted in a cell line and assays such as utertrophic response assays, and uterine thymidine incorporation assays, conducted *in vivo*, were negative.

An examination of the data from several bioassays (chronic, subchronic, developmental, and reproductive studies) employing atrazine, simazine, propazine and the major atrazine metabolites did not provide any evidence of an estrogenic effect resulting from exposure to these compounds.

Chapter 8

8 Structure Activity Relationship

Atrazine belongs to a class of compounds known as triazines in reference to the triazine ring structure that they contain. Several compounds containing triazine rings are presently registered for use in the U.S. as pesticides. Many of these pesticides (but not all) will have nitrogen atoms attached to carbon two and four. Compounds with this moiety are more appropriately referred to as "amino-s-triazines." These triazine-containing chemicals can be divided into a several classes of compounds: sulfonylurea-triazine compounds (that do not have the nitrogen at C2 and 4); alkyamino, alkythio-triazines, alkoxy-triazines and chloro-triazines. Atrazine is a chlorotriazine. The distinction between the chemical classes lies in the groups attached to the R1 position of the triazine ring (C6). Chloro-triazines will have a chlorine atom at the R1 position. The alkyamnio-triazines will have an alkyamnio moiety at R1 and the alkoxy will have an hydroxyl moiety at R1. Alkythio compounds will have an alkythio group at R1. Sulfonylurea compounds will have, at R1, a sulfonated urea moiety to which another structure, frequently a benzene ring, is attached. The structures of atrazine and several atrazine metabolites are shown in Figure 8-1. The structures of the amino-s-triazine ring itself and of chlosulfuron, a representative example of a sulfonylurea compound, are shown in Figures 8-2 and 8-3. The structures of the chloro-triazine pesticides simazine and propazine are shown in Figure 8-4.

Two-year bioassay studies using female Sprague-Dawley rats have been performed on several of the triazine compounds. As shown in Tables 8-1 and 8-2, (Only those studies that used Sprague-Dawley or CD rats, were submitted to EPA as guideline studies, and have undergone an EPA review are included in these tables) bioassay results demonstrate that the triazine ring structure, in and of itself, is not carcinogenic for mammary tumors in the female Sprague-Dawley rat (Spencer, 1991). Not shown in Tables 8-1 and 8-2 are the results from five two-year bioassays using sulfonyurea compounds. Four out of the five studies with sulfonyurea compounds were negative for carcinogenicity. The fact that four out of five sulfonyurea and three out of four alkyl amino, alkoxy, or alkythio-triazine compounds failed to induce tumors, including mammary tumors, in SD rats indicates that simply containing a triazine ring is not sufficient to render a compound carcinogenic. Chlorine at the R1 position seems to promote an increased carcinogenic potential. For example, all four of the chlorotriazine compounds are able to induce mammary tumors in female Sprague-Dawley rats. The

importance of a chlorine in the R1 position is further demonstrated by the lack of carcinogenicity seen with hydroxyatrazine (2-hydroxy-4-ethyl amino-6-isopropyl amino-s-triazine) (Chow and Hart, 1995). Hydroxyatrazine is a major plant metabolite of atrazine that differs from atrazine only in that the chlorine at R1 is replaced by a hydroxyl-group.

Table 8-1. Results of Two-Year Bioassays with Alkylamino, alkoxy, and alkythio-triazine Compounds

Chemical	Species/ Strain	Results	Reference
Cyromazine R1 - NH_2	Rat- Sprague-Dawley	Negative for oncogenicity in doses up to 3000 ppm	Blair, *et al.*, 1981
Prometryn R1- SCH_3	Rat- Sprague-Dawley	Negative for oncogenicity in doses up to 1500 ppm	Chau, *et al.*,1991
Terbutryn R1-SCH_3	Rat- CD®BR	Positive for female mammary tumors at 3000 ppm	Ciba-Geigy, 1980
Prometon R1- OCH_3	Rat- Sprague-Dawley	Negative for oncogenicity in doses up to 1000 ppm	O'Conner, *et al.*, 1988

Table 8-2. Results of Two-Year Bioassays with Chloro-triazine Compounds

Chemical	Species/ Strain	Results	Reference
Atrazine R1- Cl	Rat- Sprague-Dawley	Positive for female mammary tumors at 70 ppm	Mayhew, *et al.*, 1986
Simazine R1- Cl	Rat- Sprague-Dawley	Positive for female mammary tumors at 100 ppm	McCormick*, et al*., 1988
Propazine R1- Cl	Rat- Sprague-Dawley	Positive for female mammary tumors at 3 ppm	Jessup, 1980a
Cyanazine R1- Cl	Rat- Sprague-Dawley	Positive for female mammary tumors at 5 ppm	Bogdanffy, 1990

The lack of carcinogenicity of the hydroxyatrazine metabolite is further supported by decisions reached by the HED Metabolism Committee (MARC) which concluded in a September 29, 1995 meeting that: "For atrazine, the residues of concern for cancer dietary risk are parent and chloro metabolites" (US EPA, 1995).

Figure 8-1. Structures of Atrazine and Major Metabolites

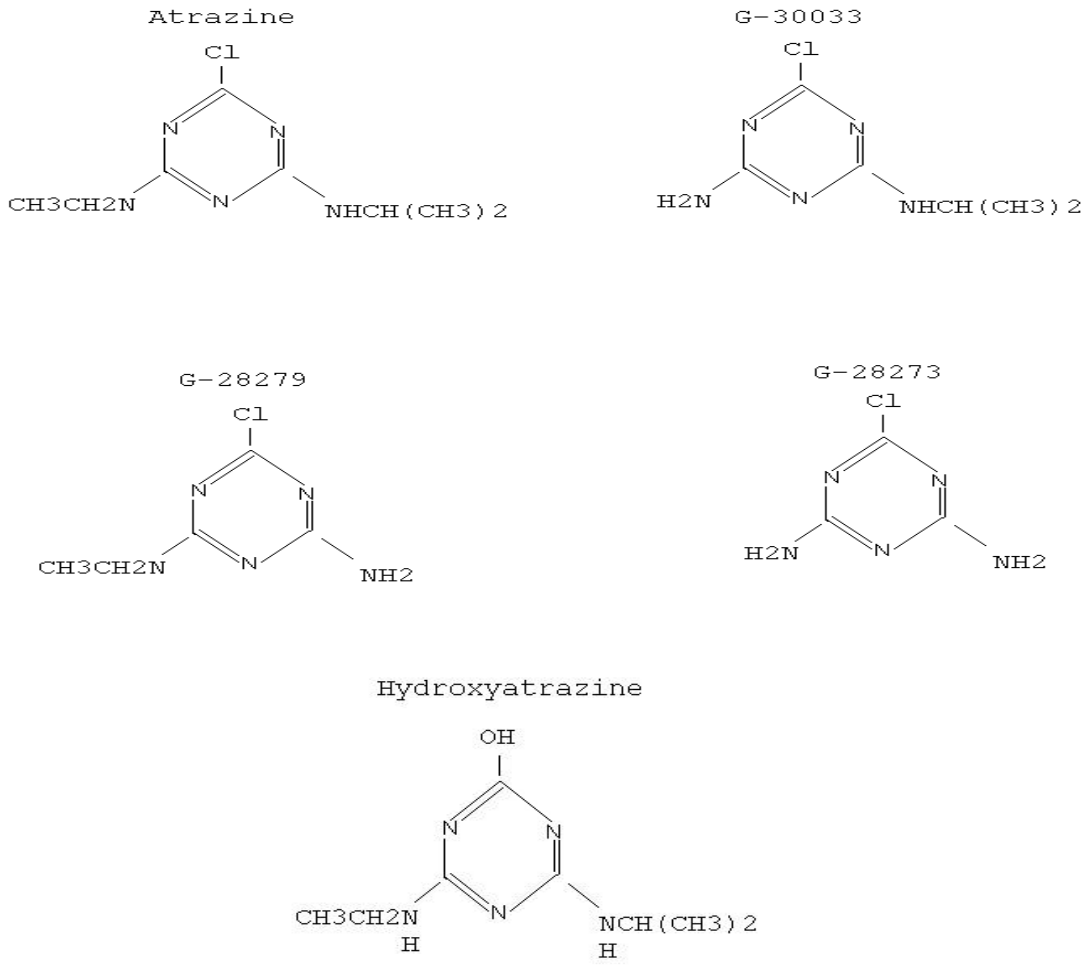
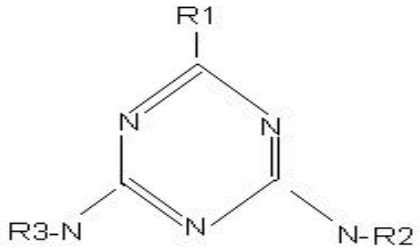


Figure 8-2. Structure of the Amino-s-Triazine Ring



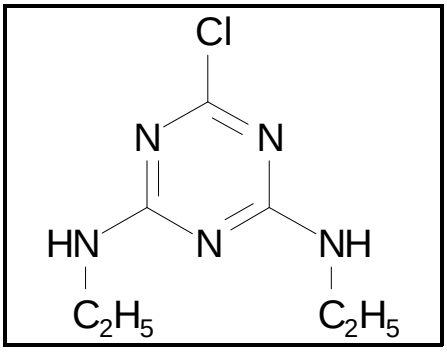
Amino-s-Triazine Ring

Figure 8-3. Structure of Chlorsulfuron

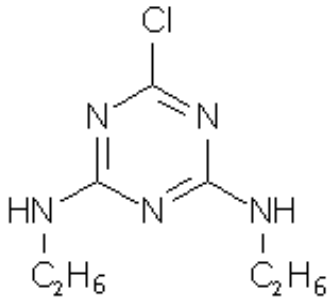
Chlorsulfuron

Figure 8-4. Structures of Simazine and Propazine

Simazine



Propazine



Of special interest when looking at structural analogues of atrazine is the functional similarity between those compounds most similar to atrazine (simazine, propazine and cyanazine) in regards to carcinogenicity. Like atrazine, all three of these compounds are positive for mammary tumors in the female Sprague-Dawley rat (see Table 8-2) and all three have been tested in two-year mouse bioassays and have been found to be negative for carcinogenicity (Hazelette and Green, 1988; Jessup, 1980b; Gellatly, 1981). Like atrazine, genotoxicity studies with simazine and propazine do not support a mutagenic potential for these compounds (see Chapter 6 - *Structural Analogs of atrazine* of this document). Mutagenicity studies with cyanazine have yielded mixed results, although the presence of the cyano group in cyanazine confounds comparison of this compound with the three other chloro-s-triazines relative to mutagenicity.

Chapter 9

9 Hormonal and Estrus Cyclicity Studies

The four previously described two-year bioassays in the SD rat clearly demonstrated that atrazine may increase mammary tumor incidence or decrease latency in the SD female. Atrazine exposure may also decrease pituitary adenoma latency in the SD female, but does not appear to alter pituitary tumor incidence. Reviews of numerous mutagenicity studies indicates that DNA damage does not appear to be contributing to these carcinogenic effects. Furthermore, results indicate that atrazine does not appear to be acting as a xenoestrogen to induce mammary tumors. The two-year bioassays with the CD-1 mouse and the F-344 rat demonstrated that there are strain/species differences in the carcinogenic effects of atrazine.

The lack of a mutagenic or exogenous estrogenic effect of atrazine, combined with the known hormonal dependence of rat mammary tumors, suggests that a perturbation of an endogenous hormonal mechanism may be responsible for the increase in mammary tumors and decreased latency of pituitary adenomas seen following atrazine exposure in the SD rat.

Mammary tumors and pituitary adenomas in the SD female are both very common occurrences. The historical control data shown in Table 9-1 demonstrates the high background tumor incidence rate for these tumor types in the female SD rat. The background tumor incidence rates of mammary tumors in SD males is <2% - much lower than in females (Lang, 1992; McMartin *et al.*, 1992). Pituitary adenomas, on the other hand, are very common in the SD male. Spontaneous pituitary adenoma rates are approximately 60% in SD males (Lang, 1992; McMartin *et al.*, 1992).

Table 9-1. Mammary Tumor and Pituitary Adenoma Historical Control Incidence Data For Sprague-Dawley Females At 24 Months

Source	Mammary Fibroadenoma	Mammary Carcinoma	Pituitary Adenoma
Pooled Charles River[1] mammary tissues from 1250 female rats in 19 studies examined	$\bar{x}$= 31.4% Range= 13.7-49.0%	$\bar{x}$= 17.68% Range= 7.1-31.4%	$\bar{x}$= 72.1% Range= 31.4-88.8%
Pooled Ciba-Geigy, Summit, N.J.[2] mammary tissues from 585 female rats in nine studies examined	$\bar{x}$= 31.3% Range= 20-43.3%	$\bar{x}$= 16.8% Range= 6.730%	$\bar{x}$=84.7% Range= 79.7-90%

[1]Lang, 1992
[2]McMartin *et al.*, 1992

It has been hypothesized that the effect of atrazine exposure is to decrease the latency of mammary cancer in the SD female rat (Stevens, 1994).

> "...it has been hypothesized that the lifetime administration of s-triazines to female Sprague-Dawley rats produces an endocrine-mediated imbalance, which causes precocious age-related changes, possibly resulting in the earlier onset or increased incidence of mammary tumors."

Implicit in this hypothesis is the belief that the high mammary tumor incidences seen in control SD females are due to the reproductive aging process in that strain. It is reasonable to assume the same mode of action proposed for mammary tumors (induction of an early onset of a state resembling reproductive aging and its associated hormonal imbalance) is also responsible for the early onset of pituitary tumors.

Pituitary tumors in the SD female are also known to be age-related (Blankenstein *et al.*, 1984; McComb *et al.*, 1984; Sandusky *et al.*, 1988). Pituitary tumors in the female rodent are known to be, at least in part, estrogen-dependent, and increased exposure to estrogens is associated with increased incidences of pituitary adenomas, hyperplasia and increased pituitary weights (Blankenstein *et al.*, 1984; McConnell, 1989a; Nelson *et al.,* 1980; Meites, 1981; McConnell, 1989b).

9.1 Rat Reproductive Aging Process

Mammary tumors are recognized as a common and expected occurrence in female SD rats, and appear to be increased primarily in aging animals. The age-related nature of the mammary tumors in female SD rats has been commented on by various authors (Cooper, 1983; Cutts and Noble, 1964). The age-related nature of the mammary tumors in SD females is illustrated by the mammary tumor incidences in control animals in the two-year oncogenicity studies described in this document. The great majority (75%) of the mammary tumors in control rats in the Thakur study (1991a) occurred when the rats were greater than one-year old. The other Thakur study (1992a) also had 75% of the mammary tumors in controls appearing after one year of age. A third two-year oncogenicity study found that 82% of the mammary tumors in control animals occurred when the animals were greater than one-year old (Morseth, 1998).

Understanding the hypothesis that the mechanism of reproductive aging in the female Sprague-Dawley leads to a high incidence of mammary tumors in females of that strain necessitates understanding the processes of reproductive aging in SD rats that are believed to lead to increased incidences of mammary tumors. At this point, a brief review and summary of these processes in the SD rat are presented. For comparisons sake, the reproductive aging process in the F-344 rat is also presented.

9.1.1 Sprague-Dawley

9.1.1.1 Alterations at the Ovary And Vagina

For reasons that are not yet completely understood, an aging female SD rat experiences a dampening of the preovulatory pituitary gonadotropin (luteinizing hormone, LH) surge (Zou, 1996; Cooper and Walker, 1979; Huang *et al.*, 1978). The preovulatory LH surge is responsible for inducing ovulation and, when the amplitude of the surge falls below a critical threshold, there is failure to ovulate. The ovaries of females with subthreshold LH surges will have reduced numbers of, or no, functional corpea lutea (CL), an increased number of secondary and antral follicles, and an increased number of follicles undergoing atresia (Smith and Conn, 1983; Cooper *et al.*, 1996; Huang and Meites, 1975). The increase in numbers of unruptured follicles results in prolonged exposure to moderately elevated levels of serum estrogens as

these follicles continue to secrete estrogen. Eventually, the unovulated follicles do undergo the process of atresia. Thus, the ovaries of aging SD rats will display increased numbers of atretic follicles. In the aging SD rat, each successive wave of follicles that undergoes atresia is replaced by a new crop that will again sustain the serum estradiol levels at a moderately elevated level.

Corpea lutea form from follicles that have ovulated and undergo the process of "lutenization" in which the granulosa cells of the follicle begin to secrete progesterone instead of estradiol. Because follicle ovulation is reduced in aging SD females, CL numbers will be reduced.

The vaginal smears of aging SD rats will consist primarily of cornified epithelial cells reflecting the tonic level of estradiol in the serum and the absence of, or low levels of, progesterone. The presence of vaginal cornification day after day as a result of the aging process is termed "constant estrus."

These changes typically begin to take place in a normally aging rat at approximately nine months of age. A typical estrous cycle in a young SD rat (< nine months in age) is four or five days in length. The first two days are diestrus, the third is proestrus and the fourth is estrus. Five-day cycles typically have an extra day of diestrus. During the aging process, cycles first become irregular and then the majority of the females transition into constant estrus. In the final months of the females life, she may become completely acyclic. Vaginal smears performed on animals at this time will indicate that the animals are in a state of extended or persistent diestrus.

The above described pattern should be considered a general rule only. The exact age at which these changes take place and their exact order may vary with the individual rat or with separate colonies of rats (LeFevere and McClintock, 1988).

9.1.1.2 Alterations at the Pituitary

Age-associated pituitary alterations have been well-described in the SD female. Pituitary weight, pituitary hyperplasia and pituitary adenomas have all been reported to be increased in aged female rats that undergo constant estrus as their primary mode of reproductive aging (SD and Long-Evans, for example) (Cónsole *et al.*, 1997; McComb *et al.*, 1984). A proliferation of the cells of the anterior pituitary that secrete prolactin (*i.e.*, lactotrophs) has been shown to be responsible for much of the increase in pituitary weight and hyperplasia seen in the aged female SD. The great majority of the pituitary adenomas seen in the aging female SD have also been found to have originated from lactotrophs (Sandusky *et al.*, 1988).

An increase in lacotroph number might be expected to result in increased serum prolactin levels in the aging rat as these cells continue to secrete prolactin. Increased serum prolactin levels are seen in aged SD females, and these increased serum prolactin levels have been correlated with increased pituitary weight, increased pituitary hyperplasia, and increased incidence of pituitary adenomas (Baird *et al.*, 1990; McComb *et al.,* 1986; van Putten *et al.*, 1988).

The alterations seen at the pituitary are believed to be due to the prolonged exposure to moderately elevated levels of serum estrogens that occur following anovulation (Nelson *et al.,* 1980; Goya *et al.,* 1990; McConnell, 1989b). Serum estrogen is known to be mitogenic to pituitary lactotrophs, and the prolonged exposure to moderately elevated serum estradiol levels likely mediates the increases in pituitary weight, hyperplasia and adenomas seen in the aging SD female. Additionally, estrogen appears to damage tuberoinfundibular neurons in the hypothalmus to inhibit production of prolactin inhibiting factor (PIF) (Sarkar *et al.,* 1982). PIF inhibits the production by the pituitary of prolactin. Thus, inhibition of its production by estrogen would have the effect of increasing prolactin production by the pituitary.

9.1.2 Fischer-344

9.1.2.1 Alterations at the Ovary and Vagina

Like the young-adult Sprague-Dawley female, the young-adult female F-344 typically displays regular four- to five-day estrous cycles. However, unlike the SD female, the ability of the aging F-344 rat to obtain an ovulatory LH surge is not compromised. Also, unlike the SD female that develops a pattern of constant estrus within the first year of life, the regular ovarian cycles present in the F-344 give way to a pattern of repetitive pseudopregnancies (Huang *et al.,* 1978; McConnell, 1989a; Estes, 1982). In this condition, ovulation occurs periodically and the newly formed CL are maintained for extended periods (10 to16 days). There are two distinct changes in the pattern of hormone secretion that are present in the pseudopregnant female - increases in serum prolactin and serum progesterone. First, there are two, daily prolactin peaks that occur just before lights on and just before lights out. In response to the elevated prolactin levels, the ovarian CL are maintained. The functional CL are responsible for the elevated levels of progesterone present in these animals. In addition to CL, the ovaries of an aging F-344 will contain a moderate number of antral and secondary follicles, and a moderate number of atretic follicles. Again, these findings are in contrast to the SD female that will contain few, if any CL, many secondary and antral follicles, and many atretic follicles. Also, in contrast to the constant estrus female that is characterized by uninterrupted cornification (typical of the aging SD rat), the vaginal cytology of the pseudopregnant female is primarily leukocytic. This vaginal smear pattern is similar to that seen during the diestrus period of the estrous cycle and this pattern may be referred to as "persistent diestrus," although the term “repetitive pseudopregnancy” is also used, and is the more descriptive term. As was the case with the SD, in the final months of the F-344 females life, she may become completely acyclic. The same disclaimer about variability in SD rat reproductive aging process applies to the F-344. The description above can only be seen as a generality.

9.1.2.2 Alterations at the Pituitary

The alterations seen in the pituitary of the female SD are not seen in the aging F-344 rat. The incidence of pituitary adenomas in female F-344 rats at two-years of age is much less than the incidence of pituitary adenomas in female SD rats at two-years of age (Sandusky *et al.*, 1988). Increases in pituitary weights and pituitary hyperplasia are also not commonly seen in the aging F-344 female.

9.1.3 Summary of the Reproductive Aging Process in SD and F-344 Rats

Characteristics of reproductive aging in the female SD have been throughly studied (Cooper, 1983; Cooper *et al.*, 1996; LeFevere and McClintock, 1988; Meites *et al*., 1980). The appeal of hypothesizing that these events may result in mammary carcinogenicity comes from the prolonged exposure to estrogen and prolactin that results from these events. As noted above -- the increased days spent in estrus in the aging SD female appears to be related to the attenuated LH surge, which results in numerous unovulated ovarian follicles constantly producing estrogen. The prolonged exposure to estrogen acts at the pituitary to stimulate increased prolactin production.

The role of prolonged or inappropriate exposure to estrogen and prolactin in mammary carcinogenicity in rats has been well-established (Nandi *et al.*, 1995; Russo *et al.*, 1990; Cutts and Noble, 1964; Meites, 1972). It is reasonable to hypothesize that the prolonged exposure to estrogen and prolactin that results from the normal reproductive aging process in SD females may be responsible for the high levels of mammary tumors normally seen in this strain. This hypothesis was first advanced as long ago as 1966 (Durbin *et al.,* 1966).

The mode of reproductive aging in the F-344 rat is different from that of the SD female. Reproductive aging in the F-344 does not result in prolonged estrogen exposure. Prolactin levels are increased in the aging F-344, as are progesterone levels.

A summary of the different modes of reproductive aging in the SD, and F-334 is shown in Figure 9-1.

Figure 9-1. Summary of the Reproductive Aging Process in Different Rat Strains

F-344

- Normal cycle is a four to five days with 25% of the time spent in estrus, 25% spent in proestrus and 50% spent in diestrus;
- Reproductive aging becomes evident at approximately 12 months;
- Reproductive aging is characterized by increased prolactin surges that leads to maintenance of the corpea lutea;
- There is an increase in the days spent in diestrus, and increased exposure to progesterone.
- In very aged animals, acyclicity is common

Sprague-Dawley, Long Evans, Wistar[1]

- Normal cycle is a four to five day cycle with 25% of the time spent in estrus, 25% spent in proestrus and 50% spent in diestrus;
- Reproductive aging becomes evident at approximately nine to 12 months;
- Reproductive aging is characterized by decreased gonadotropin surges that leads to maintenance of primary, secondary and antral ovarian follicles;
- An irregular cycling pattern develops followed by an increase in the days spent in estrus, and prolonged exposure to estrogen;
- Pituitary alterations such as increase in pituitary weight, increases in pituitary hyperplasia and pituitary -adenomas become common as the animal ages;
- Acyclicity develops in the final months of life
- Normal cycling ➜ irregular cycles ➜ prolonged estrus ➜ acyclicity
  (begins around 5-6 weeks old) *(occurs in the last few months of life ≥ 21 months of age)*

[1]*There may be temporal differences between and among strains*

9.1.4 Strain Differences in Reproductive Aging and Mammary Tumors

Evidence supporting the hypothesis that the normal reproductive aging process in the SD female leads to mammary tumors in this strain can be found by comparing the hormonal environment associated with reproductive aging and mammary tumor historical control values in the F-344 to the SD. Direct experimental evidence implicating the reproductive aging process of the SD is also available and is discussed below.

9.1.4.1 The Different Hormonal Environments of the SD and F-344 Rats During Reproductive Aging

The hormonal environment of the normally aging SD female is one of increased exposure to both estrogen and prolactin. The hormonal environment of the normally aging F-344 rat is one of increased exposure to prolactin and progesterone.

Increased or prolonged exposure to estrogen or prolactin is implicated in mammary carcinogenesis. Female rats that have been implanted subcutaneously with pellets of estrogen have very high incidences of mammary tumors and an earlier onset of those tumors than would be expected (Cooper, 1983; Cutts and Noble, 1964). Animals that have had pituitaries implanted under their renal capsule[2] or have been given lesions in the arcuate nucleus-median eminence of the hypothalmus[3] also have high incidences and earlier onset of mammary tumors (Welsch *et al.*, 1970a and 1970b). In contrast, increased or prolonged exposure to progesterone does not increase the incidence or decrease the onset of mammary tumors in the rat (Welsch, 1987).

[2]Implantation of pituitaries under the renal capsule results in high levels of prolactin secretion from the implanted pituitaries.

[3]Lesions in this area of the brain result in production of high levels of pituitary prolactin.

Increased exposure to either estrogen or prolactin increases mammary tumor risk in the rat, while increased exposure to progesterone - in the absence of increased exposure to either estrogen or prolactin - does not increase mammary tumor risk. Progesterone can, in fact, be anti-carcinogenic. The ratio of estrogen to progesterone is an important aspect to consider when examining mammary carcinogenesis. Progesterone has been shown to "oppose" estrogen. This means that high levels of progesterone can counteract high levels of estrogen and reduce the increased risk of mammary cancer seen with high serum estrogen levels. The molecular mechanism by which progesterone opposes estrogen's actions appears to be by down-regulation of mammary, hypothalamic and pituitary estrogen receptors (Mauvais-Jarvis *et al.,* 1987; Libertun *et al.,* 1979; Cho *et al.,* 1993; Brann *et al.,* 1988).

9.1.4.2 Different Mammary Tumor Historical Control Values in the SD and the F-344

The Thakur studies described above show the low incidence of mammary tumors in control F-344 females. Charles River Laboratories has reported relatively low historical control rates for female F-344 mammary carcinomas (1.5%) and fibroadenomas (12%) while control rates for other types of mammary tumors are even lower (Lang, 1990). A recent report of the spontaneous neoplasms seen in the National Toxicology Program (NTP) two-year bioassays confirms a low mammary carcinoma rate for F-344 females (3.1%), but finds that the fibroadenoma incidence rates are now 41.2% after having increased from 28% in 1985 (Haseman *et al.,* 1998). Hazelton Labs reports the historical control incidence of F-344 female mammary carcinomas (adenocarcinomas and carcinomas combined) as 3.8% and the rate of fibroadenomas as 14.9% (Hazelton Labs, 1994). The values for background mammary tumors in the F-344 are much lower than in the SD (with the exception of adenocarcinomas in the NTP studies). These differences in background mammary tumor incidences could be due the different mechanisms of reproductive aging.

Historical control data from rat strains other than the SD and F-344 confirms that animals whose predominant mode of reproductive aging is constant estrus have higher background mammary tumor incidences than F-344. Table 9-2 displays the mode of aging in several strains of rats and the historical control incidence of mammary tumors in each strain.

Table 9-2. Relationship of Reproductive Aging and Mammary Tumor Incidence In Various Rodent Strains

Strain/ Species	Reproductive Aging	Spontaneous Mammary Tumor Incidence	References
SD/rat	Constant estrus	~30% fibroadenoma ~12% carcinoma	Aging -- Estes, 1982 Tumors -- Lang, 1992
Wistar/rat	Constant estrus	~25% fibroadenoma ~13% carcinoma	Aging -- Mora *et al.*, 1994a; Mora *et al.*,1994b Tumors -- Walsh and Poteracki, 1994
Long-Evans Hooded/rat	Constant estrus	~50% with either fibroadenoma or carcinoma	Aging -- Estes, 1982 Tumors -- Cooper, 1983
F-344/rat	Pseudopregnancy	~12% fibroadenoma ~2% carcinoma	Aging -- Estes, 1982 Tumors -- Lang, 1990

9.1.5 Studies in Which Premature Aging Was Artificially Delayed or Induced and How Mammary Tumor Incidences Were Affected

Experimental evidence is available that examines the relationship between reproductive aging and mammary carcinogenesis.

9.1.5.1 Mammary Tumor Onset Is Delayed in Rats Fed a Diet Supplemented with L-tyrosine

Supplementing the diet of the female Long-Evans rat with the amino acid L-tyrosine will delay the onset of constant estrus (Cooper and Walker, 1979). These studies showed that approximately 95% of the female Long-Evans rats on regular diets will be in constant estrus by 13 months of age while 60% of the females fed L-tyrosine will still be cycling normally at 16 months of age. Mammary tumor onset in the Long-Evans females fed L-tyrosine supplemented diets was also delayed. Mammary tumor incidence in control LE females was 67% by 19 to 21 months of age. No female fed an L-tyrosine supplemented diet had a mammary tumor at 19 to 21 months. Even at 25 months of age, L-tyrosine supplemented animals tumor incidence rates are only 25%.

The implication from these findings is that the delay in the appearance of mammary tumors is due to the delay in the onset of constant estrus (*i.e.*, the delay in reproductive aging).

9.1.5.2 Mammary Tumor Onset Is Accelerated in Females Receiving High Levels of Estrogen Through Implants

The hormonal state of constant estrus rodents is one of moderately elevated levels of serum estrogen and low levels of serum progesterone (Huang *et al.,* 1978). Ovariectomizing female rodents and then exposing them to a chronically high level of estrogen through subcutaneous implants of silastic tubing containing estrogen will mimic the hormonal state seen in constant estrus females and these females will develop mammary tumors much earlier than control animals (Cooper, 1983). The mammary tumor incidence rate at 12 months for Long-Evans females who were OVX at four months of age and given implants is 100% while the control rate is less than 5%.

9.1.5.3 Mammary Tumor Onset Is Accelerated in Females Whose Reproductive Aging Has Been Accelerated by Exposure to Constant Light

Rats exposed to constant light (rather than a light cycle such as 12 hours light/12 hours dark or 14 hours light/10 hours dark) show an earlier onset of constant estrus. Such rats show an increased incidence and earlier onset (latency was decreased by 34% compared to animals on light/dark cycles) of mammary tumors- primarily adenocarcinomas (Molina *et al.,* 1981).

9.1.6 The Correlation Between Increased Days in Estrus and Mammary Tumors in a Two-Year Bioassay

Estrous cycle evaluations were performed in a two-year bioassay using SD females (tumor data from this study - Morseth, 1998 - is discussed in section 5.3 while estrous cycle data is discussed below under section 9.2.3 – Results of Estrous Cycle Measurements in the SD rat - Thakur, 1999). These evaluations showed that the percent of total days spent in estrus was significantly higher in animals with mammary tumors than in those without. Tumor latency was also reduced in animals that spent a longer time in estrus.

9.1.7 Summary and Discussion of the Hypothesis that Mammary

Tumors Are Induced by the Reproductive Aging Process

The hypothesis that the mode of reproductive aging in some strains of rat (including the SD) may contribute to mammary tumor formation has received attention for many years. The hypothesis is well established in that:

- Female SD rats undergo a mode of reproductive aging that results in extended periods of estrus;
- These periods of extended estrus result in prolonged exposure to moderately elevated serum estrogen derived from unovulated ovarian follicles;
- Serum estrogen acts at the pituitary to increase prolactin secretion;
- Increased exposure to estrogen and prolactin increases the risk of mammary carcinogenicity in rats.

The connection between the mode of reproductive aging and mammary carcinogenicity can be confirmed by examining rodent strains in that reproductive aging involves induction of a primarily constant estrus state. The estrous cycles of the SD, Wistar, and Long-Evans strain of rat all progress through a state of constant estrus as the animals age and all these strains have high background incidences of mammary tumors. The estrous cycle of the aging F-344 strain of rat is primarily one of pseudopregnancy and background mammary tumor incidences in the F-344 rat are low.

Experimental evidence showing the relationship between reproductive aging and mammary carcinogenicity is provided by studies in which the reproductive aging process in rats was either delayed or accelerated resulting in mammary tumor onset being delayed or accelerated. Delaying the onset of reproductive aging by feeding rats diet supplemented with L-tyrosine delayed the onset of mammary tumors. Accelerating the reproductive aging process by creating a hormonal state that mimics the hormonal state of reproductive aging results in mammary tumors appearing at a much earlier age. Accelerating the reproductive aging process by exposure to constant light also accelerates the onset of mammary tumors.

9.2 The Hypothesis that Atrazine Exposure Induces an Early Onset of Attenuated LH Surge, Increased Days In Estrus, and Prolonged Exposure to Estradiol

It is postulated that atrazine induces an early onset or increased incidence of mammary tumors by inducing an early occurrence of an attenuated LH surge, increased days in estrus, and prolonged exposure to estrogen and prolactin. The evidence testing this hypothesis can be found in six areas:

- Examination of the time-to-tumor in atrazine exposed rats;
- Examination of serum estradiol levels in atrazine exposed rats;
- Examination of alterations in the ovary (early onset of decreased CL, increased antral, secondary and atretic follicles) and vagina (early onset of increased days in estrus) of atrazine exposed rats;
- Examination of the pre-ovulatory LH surge in atrazine exposed rats.
- Examination of pituitary alterations in atrazine-exposed rats.
- Examination of mammary gland alterations that are indicative of prolactin exposure in atrazine-exposed rats.

9.2.1 Time-to-Tumor

Examination of the onset time of mammary tumors in female SD rats in the two-year bioassays (excluding the Mayhew study that is not amenable to this type of analysis) shows that atrazine exposure did induce an earlier onset of carcinomas (Tables 9-3 and 9-4). An early onset of fibroadenomas was less evident, but nevertheless, there also appeared to be an early of onset of this tumor type. Onset time was determined in these studies by examining the histopathology data and the clinical observations and correlating the appearance, by palpation, of each mass that was subsequently confirmed by histopathology to be a mammary tumor. Analysis of the onset time for mammary tumors in the two-year bioassays with structurally related s-triazines simazine and propazine, are also considered (Tables 9-5 and 9-6). Additional data concerning onset time can be obtained from a one-year study submitted by the registrant (Pettersen and Turnier, 1995).

Table 9-3. Time to Mammary Tumor in the Female SD Rat- Thakur (1992a) Terminal Sacrifice Protocol

	Dose (mg/kg/day) and Tumor Type					
	0 Fib	**3.79 Fib**	**23.01 Fib**	**0 Carc**	**3.79 Carc**	**23.01 Carc**
$\bar{x}$ wk of appear	76.4	76.1	72.7	78.9	72.5	65.4
$\leq$52wk	2/35 (5.7%)	1/27 (3.7%)	3/39 (7.7%)	0/14 (0%)	3/11 (27.3%)	6/18 (33.3%)
p-value	0.379	0.598	0.552	0.024*	0.072	0.021*
53-78 wk	16/35 (45.7%)	15/27 (55.6%)	18/39 (46.2%)	8/14 (57.1%)	3/11 (27.3%)	5/18 (27.8%)
p-value	0.414	0.304	0.578	0.099	0.138	0.094
79-104 wk	17/35 (48.6%)	11/27 (40.7%)	18/39 (46.2%)	6/14 (42.9%)	5/11 (45.5%)	7/18 (38.9%)
p-value	0.15	0.361	0.510	0.423	0.608	0.553

Notes: The dose shown is in mg/kg/day.

Fib= fibroadenomas. Carc= carcinomas and adenomas combined.

*$p \leq 0.05$

Table 9-4. Time to Mammary Tumor in the Two-year Morseth (1988) Study

	Dose (mg/kg/day) and Tumor Type									
	0 Fib	**1.5 Fib**	**3.1 Fib**	**4.2 Fib**	**24.4 Fib**	**0 Carc**	**1.5 Carc**	**3.1 Carc**	**4.2 Carc**	**24.4 Carc**
≥wk of appear	76.1	72.4	73.7	73.3	76.3	72.6	77.2	78.6	64.4	64.8
≤52wk	0/15 (0%)	1/18 (5.6%)	3/26 (11.5%)	1/26 (3.8%)	1/22 (4.5%)	1/11 (9.1%)	2/15 (13.3%)	0/14 (0%)	2/10 (20.0%)	6/23 (26.1%)
p-value	0.549N	0.546	0.244	0.634	0.595	0.047*	0.619	0.440N	0.462	0.252
53-78 wk	9/15 (60.0%)	11/18 (61.1%)	13/26 (50.0%)	14/26 (53.8%)	9/22 (40.9%)	5/11 (45.5%)	6/15 (40.0%)	7/14 (50.0%)	6/10 (60.0%)	7/23 (30.4%)
p-value	0.104N	0.614	0.386N	0.479N	0.211N	0.110N	0.548	0.570	0.410	0.315
79-104 wk	6/15 (40.0%)	6/18 (33.3%)	10/26 (38.5%)	11/26 (42.3%)	12/22 (54.5%)	5/11 (45.5%)	7/15 (46.7%)	7/14 (50.0%)	2/10 (20.0%)	10/23 (43.5%)
p-value	0.092	0.486N	0.590N	0.575	0.297	0.526	0.632	0.570	0.221N	0.600

NOTEs: The dose shown is in mg/kg/day.

Fib= fibroadenomas. Carc= carcinomas and adenomas combined.

Trend for the dose is shown in the control column. "N" indicates that the trend is negative; otherwise trend is positive.
*$p \leq 0.05$

Table 9-5. Time to Mammary Tumor with Simazine in SD rats (McCormick *et al.*, 1988)

	Dose (mg/kg/day) and Tumor Type							
	0 Fib	**0.52 Fib**	**5.3 Fib**	**63.1 Fib**	**0 Carc**	**0.52 Carc**	**5.3 Carc**	**63.1 Carc**
$\bar{x}$ wk of appear	86	81.7	89.6	68.8	83.4	80.7	82.1	65.9
≤52wk	1/23 (4.3%)	1/28 (3.6%)	0/18 (0%)	6/42 (14.3%)	2/18 (11.1%)	3/17 (17.6%)	2/20 (10.0%)	13/44 (29.5%)
p-value	0.029*	0.704	0.561	0.212	0.026*	0.472	0.656	0.110
53-78 wk	6/23 (26.1%)	7/28 (25.0%)	3/18 (16.7%)	26/32 (61.9%)	3/18 (16.6%)	4/17 (23.5%)	4/20 (20.0%)	18/44 (40.9%)
p-value	0.000**	0.590	0.370	0.006**	0.013*	0.466	0.563	0.059
79-104 wk	16/23 (69.6%)	20/28 (71.4%)	15/18 (83.3%)	10/42 (23.8%)	13/18 (72.2%)	10/17 (58.8%)	14/20 (70.0%)	13/44 (29.5%)
p-value	0.000**N	0.563	0.260	0.000** N	0.000** N	0.316 N	0.583 N	0.003** N

NOTEs: The dose shown is in mg/kg/day.

Fib= fibroadenomas. Carc= carcinomas and adenomas combined.

Trend for the dose is shown in the control column. "N" indicates that the trend is negative; otherwise trend is positive.

*p≤0.05; **p≤0.01.

Table 9-6. Time to Mammary Tumor with Propazine in SD rats (Jessup, 1980a)

	Dose (mg/kg/day) and tumor type							
	0 Fib	**0.2 Fib**	**6.4 Fib**	**68 Fib**	**0 Carc**	**0.2 Carc**	**6.4 Carc**	**68 Carc**
$\bar{x}$ wk of appearance	84	80.6	77.6	75.7	90.2	77.8	85.2	77.28
≤52wk	1/21 (4.8%)	0/21 (0%)	1/17 (5.9%)	0/19 (0%)	0/7 (0%)	0/15 (0%)	0/13 (0%)	2/25 (8.0%)
p-value	0.406	0.500N	0.701	0.525N	0.170	1.000	1.000	0.605
53-78 wk	4/21 (19.0%)	10/21 (47.6%)	8/17 (47.1%)	13/19 (68.4%)	2/7 (28.6%)	8/15 (53.3%)	4/13 (30.8%)	11/25 (44.0%)
p-value	0.005**	0.050	0.067	0.002**	0.430	0.268	0.664	0.389
79-104 wk	16/21 (76.2%)	11/21 (52.4%)	8/17 (47.1%)	6/19 (31.6%)	5/7 (71.4%)	7/15 (46.7%)	9/13 (69.2%)	12/25 (48.0%)
p-value	0.010*N	0.099N	0.065N	0.006** N	0.220N	0.268N	0.664N	0.254N

NOTEs: The dose shown is in mg/kg/day.

Fib= fibroadenomas. Carc= carcinomas and adenomas combined.

Trend for the dose is shown in the control column. "N" indicates that the trend is negative; otherwise trend is positive.

*p≤0.05; **p≤0.01.

9.2.1.1 Thakur, 1992a

The mean week of fibroadenoma onset in controls in this study was 76.4 weeks. The mean week of fibroadenoma onset in the 3.79 mg/kg/day and 23.01 mg/kg/day groups was 76.1 and 72.7, respectively. The mean week of onset for carcinomas was 78.9 in controls while the mean week of onset for carcinomas and adenomas in the 3.79 mg/kg/day and 23.01 mg/kg/day groups was 72.5 and 65.4.

The percentage of carcinomas and adenomas occurring in the first year of the study in controls was 0% while at 3.79 mg/kg/day and 23.01 mg/kg/day 27.3 and 33.3% of the carcinomas appeared in the first year of the study.

9.2.1.2 Thakur, 1991a

The percentage of fibroadenomas occurring in the first year of the study in the controls was 16.7. At 4.23 mg/kg/day and 26.23 mg/kg/day the percentage was 0 and 20%.

The percentage of carcinomas occurring in the first year of the study was 0 in controls and 33% at 4.23 mg/kg/day and 50% at 26.23 mg/kg/day.

9.2.1.3 Morseth, 1998

The mean week of fibroadenoma onset in controls in this study was 76.1 weeks. The mean onset in the 1.5, 3.1, 4.2 and 24.4 mg/kg/day groups was 72.4, 73.7, 73.3 and 76.3 weeks, respectively. The mean week of onset for carcinomas and adenomas in controls was 72.6 while the mean week of onset for the 1.5, 3.1, 4.2 and 24.4 mg/kg/day groups was 77.2, 78.6, 64.4 and 64.8, respectively.

The percentage of fibroadenomas in the control group that occurred in the first year of the study was 0. The percentage of fibroadenomas that occurred in the first year of the study in the 1.5, 3.1, 4.2 and 24.4 mg/kg/day groups was 5.6, 11.5, 3.8 and 4.5, respectively. The percentage of carcinomas and adenomas that occurred in the first year of the study was 9.1 and the percentage that occurred in the 1.5, 3.1, 4.2 and 24.4 mg/kg/day groups was 13.3, 0, 20, 26.1, respectively.

There was an increased mammary tumor incidence at the 53 week interim sacrifice, which also indicates and earlier tumor onset. There were no fibroadenomas at the 53 week sacrifice in the 20 females of the control group, but in the dose groups the fibroadenoma rates were 1/20, 2/19, 2/20, 1/20 in the 1.5, 3.1, 4.2 and 24.4 mg/kg/day groups, respectively.

9.2.1.4 Pettersen and Turnier, 1995

The percentage of animals with fibroadenomas in the controls was 5.9 and the percentage that occurred at the 0.8, 1.7, 2.8, 4.1 and 23.9 mg/kg/day dose groups was 5.9, 5.9, 0, 8.8, and 8.8, respectively. The percentage of animals with carcinomas and adenomas in the control group was 2.9 and the percentage that occurred at the 0.8, 1.7, 2.8, 4.1 and 23.9 mg/kg/day dose groups was 2.9, 2.9, 5.9, 5.9 and 14.7, respectively.

9.2.1.5 McCormick *et al.*, 1988 (simazine)

The mean week of fibroadenoma onset in controls in this study was 86 weeks. The mean onset in the 0.52, 5.3, and 63.1 mg/kg/day groups was 81.7, 89.6 and 68.8 weeks, respectively. The mean week of onset for carcinomas and adenomas was 83.4 for controls and 80.7, 82.1 and 65.9 for the 0.52, 5.3, and 63.1 mg/kg/day groups, respectively.

The percentage of fibroadenomas occurring in the first year of the study in controls was 4.3 and the percentage of the 0.52, 5.3, and 63.1 mg/kg/day groups occurring in the first year of the study was 3.6, 0, and 14.3, respectively. The percentage of carcinomas occurring in the first year of the study in controls was 11.1 and the percentage occurring in the dose groups was 17.6, 10 and 29.5, respectively.

9.2.1.6 Jessup, 1980a (propazine)

The mean week of fibroadenoma onset in controls in this study was 84 weeks. The mean fibroadenoma onset in the 0.2, 6.4 and 68 mg/kg/day groups was 80.6, 77.6 and 75.7 weeks, respectively. The mean week of carcinoma onset in controls was 90.2 and the mean week of onset for the 0.2, 6.4 and 68 mg/kg/day groups was 77.8, 85.2 and 77.3, respectively.

The percentage of fibroadenomas occurring in the first year of the study was 4.8 and the percentage of fibroadenomas occurring in the first year in the 0.2, 6.4 and 68 mg/kg/day group was 0, 5.9, and 0, respectively. The percentage of carcinomas that occurred in the first year of the study in controls was 0 and the percentage occurring in the dose groups was 0, 0, and eight, respectively.

9.2.2 Conclusions Of The Time-to-Tumor Data

The data from four two-year and one-year bioassays demonstrate that there is a decreased time-to-tumor for mammary tumors in the female SD rat following atrazine exposure. The earlier onset is more evident for carcinomas and adenomas than for the fibroadenomas. The mean week of carcinoma onset drops with atrazine exposure in both of the two-year studies with atrazine for which mean week of tumor onset is applied and also drops with exposure in the two-year bioassays with simazine and propazine.

The percentage of carcinomas occurring in the first year of the study is increased with exposure in three two-year bioassays with atrazine and is also increased in a one-year bioassay. Both the simazine and propazine studies showed an increase in percent of carcinomas and fibroadenomas occurring in the first year in dosed animals compared to controls.

9.2.3 Alterations in the Ovary and Vagina

9.2.3.1 Sprague-Dawley

Aging SD rats normally undergo alterations in their estrous cycles. These alterations are described above under the section 9.1 and are summarized in Figure 9.1. In brief, the alterations in a normally aging SD female rat are an increase in the percentage of days of the estrous cycle spent in estrus from 25% prior to nine months old to >40% after nine months old. The increase in days spent in estrus occurs at the expense of days spent in diestrus and proestrus.

Eldridge *et al.* (1993a) examined estrous cycle alterations in female SD rats in response to atrazine exposure. Care should be taken when reading this document not to confuse the study that is referred to as Thakur, 1991a and the study referred to as Eldridge, 1993a. The Thakur and Eldridge studies are, in fact, the same studies. The hormone and estrous cycle evaluation are referred to in this document as Eldridge, 1993a while the animal necropsy and histopathology portions of this study is referred to as Thakur, 1991a.

Histomorphologic evaluation of the ovaries and other tissues (including the vagina and mammary gland) from the Thakur/Eldridge studies was performed and is referred to as McConnell, 1995 in this document. Only the histomorphologic evaluation of the ovaries and the vagina are discussed in this section of the document. Discussed under section 9.2.5 is the histomorphic evaluation of the mammary gland of atrazine-exposed rats.

Therefore, the three citations -- Thakur, 1991a; Eldridge, 1993a; and McConnell, 1995 -- refer to different analysis performed on the same group of SD or F-344 rats as part of the same study.

Additionally, estrous cycles were evaluated in one month, six month and two-year studies -- Morseth, 1996a, 1996b, 1998). The protocol and measurements for estrous cycles in these studies are described below.

9.2.3.2 Fischer-344

Aging F-344 rats normally undergo alterations in their estrous cycles. These alterations are described above and are summarized in Figure 9.1. In brief, a state of pseudopregnancy or persistent diestrus becomes a common occurrence in F-344 rats as they progress beyond approximately nine months of age. The ovaries of animals in persistent diestrus contain CL, a moderate number of secondary and antral follicles, and moderate numbers of atretic follicles.

The same study that examined serum hormone levels in F-344 (Eldridge *et al.*, 1993b) also examined estrous cycle alterations in response to atrazine exposure. Doses and sacrifice schedules for these two studies were previously described. Histomorphologic evaluation of the ovaries and other tissues (including the vagina, mammary gland, and pituitary) from the Thakur/Eldridge studies was performed and is referred to as McConnell, 1995 in this document.

9.2.3.3 Protocol For Examination of the Ovaries and Estrous Cycle Measurements

Thakur Studies. Two separate studies were conducted: one with SD females and one with F-344 females. In both studies, 10 animals per dose group were sacrificed after approximately one, three, nine, 12, 15, and 18 months of exposure to atrazine. Two weeks before scheduled sacrifice daily vaginal smears were performed. The smears were examined for the presence of keratinized epithelium, nucleated epithelium and leucocytes. The presence of well-defined keratinized cells was taken to be indicative of estrus. The presence of leucocytes in the vaginal smears indicated diestrus and the presence of moderate- to- dense nucleated epithelium and moderate cornified epithelium was taken to be indicative of proestrus. Following the 14 days of smear collection animals were sacrificed at their next proestrus day. Thus, some animals were sacrificed on their scheduled sacrifice date (on day 15 after vaginal smears were begun) while others were sacrificed in the week following their scheduled sacrifice date (on days 16 to 21 following initiation of vaginal smears, depending on when their next proestrus phase occurred). Any animals that had not had a proestrus phase by day 21 after initiation of vaginal smears was sacrificed on day 21. Surviving animals were sacrificed at 24 months, again following the procedure described above whereby each animal is sacrificed on a proestrus phase, if possible.

Results of the estrous cycle evaluations were examined at: the dose groups at each individual timepoint compared to controls; for trend within dose at each individual timepoint; and the effect of treatment over time. These are the same parameters for which the hormone measurements were analyzed.

The ovaries of all females in these studies (both the SD and F-344 studies) were examined by standard histomorphologic techniques for several parameters including (but not limited to): absence of CL; reduced number of CL; presence of secondary and antral follicles; presence of atretic follicles.

The parameters of absence of CL and reduced number of CL were graded as simply being "present" or "absent." The parameters of secondary, antral follicles, and atretic follicles were graded on a scale of zero to five with zero indicating the parameter was absent and one to five indicating the parameter was present - the higher the number the more of the follicles were present.

Morseth, 1996a (one-month study). Vaginal smears were performed after seven days of treatment and continued daily for three weeks. The criteria for evaluation and classification of vaginal smears was similar to that followed in the Thakur studies.

Morseth, 1996b (six-month study). Vaginal smears were performed on the first day of treatment and continued daily for 14 consecutive days every four weeks. Thus, an animal was smeared in cycles of two weeks smearing followed by two weeks non-smearing. This cycle continued throughout the study. The criteria for evaluation and classification of vaginal smears was similar to that followed in the Thakur studies.

Morseth, 1998 (two-year study). Estrous cycles evaluations were performed on all intact females in this study (80 per dose group). Vaginal smears were performed for two consecutive weeks every two weeks starting on study week one. Thus, animals had two weeks of smears followed by two weeks without smears. The criteria for evaluation and classification of vaginal smears was similar to that followed in the Thakur studies. Estrous cycle data for the first 46 weeks of the study have been analyzed.

9.2.3.4 Results of Ovarian Histomorphology and Estrous Cycle Measurements in the SD Rat (McConnell, 1995 and Eldridge, 1993a)

The only study that performed histomorphologic examination of the ovaries of atrazine-treated SD rats was the Thakur, 1991a study. The histomorphologic data are presented in McConnell, 1995 while the vaginal smear data (for determination of phase in estrous cycle) are presented in Eldridge, 1993a.

Ovarian Histomorphologic Examination (McConnell, 1995). An early onset of anovulation is seen as early as three months following atrazine exposure. After three months of exposure the number of control animals with an absence of CL was zero of ten while the number of treated animals with no CL was one of ten at the 4.23 mg/kg/day group and two of ten in the 26.23 mg/kg/day group. This small increase in animals with no CL indicates that incidence of anovulation was increased in the dose groups. The fact that no CL were seen at all indicates that ovulation had not even occurred in the recent past, as even the CL from previous cycles in which ovulation did occur had regressed (*i.e*., become corpus albicans). The number of animals at three months with reduced numbers of CL was increased from two in the control group to three at 4.23 mg/kg/day and three at 26.23 mg/kg/day. Reduced CL number also indicates anovulation, but in these animals ovulation likely did occur in the recent past as CL of some type were present. The antral follicle group mean graded index (antral follicle score on the above described scale of

zero to five divided by the number of animals in the group) was 1.2 for control, 2.1 for 4.23 mg/kg/day and 2.2 for 26.23 mg/kg/day. This increase in antral follicles again indicates anovulation.

Evidence of early onset of anovulation was also seen at nine months. After nine months of exposure the number of control animals with an absence of CL was six of ten while the number of treated animals with no CL was seven of ten at the 4.23 mg/kg/day group and ten of ten in the 26.23 mg/kg/day group. The antral follicle group mean graded index was 2.6 for control, 3.1 for 4.23 mg/kg/day and 3.8 for 26.23 mg/kg/day.

By 12 months of exposure the ovarian histomorphology indicated that nearly all the animals in all dose groups were not ovulating.

Estrous Cycle Evaluations (Eldridge, 1993a). Appendix Table 8 displays the results of the estrous cycle analyses done with the SD rat in Eldridge, 1993a.

The estrous cycle results seen in this study are what would be expected in the SD strain of rat. The percent days spent in estrus in control animals increases as the animals age at the expense of days spent in diestrus, and proestrus. Linear regression analysis indicated that the decrease in diestrus and proestrus and the increase in estrus over the period of one to18 months was statistically- significant at $p<0.01$. The % days in both diestrus and proestrus are also decreased in a statistically-significant manner ($p<0.01$) in both dose groups for the period from one to 18 months. Both dose groups showed a significant ($p<0.01$) trend toward an increased % days spent in estrus from months one to 18. Such an increase was seen in controls, is to be expected, and did not appear to be altered by exposure to atrazine.

The percent days of the cycle spent in estrus was dramatically increased in dosed animals compared to controls at nine, 12 and 18 months. Females in the 4.23 and 26.23 mg/kg/day groups at nine months spent an average of 34.3 and 44.8% of their days in estrus, respectively ($p<0.05$ at 70 and $p<0.01$ at 400 ppm). This is compared to 24% spent in estrus at the controls. The dose-related trend, as determined using a Terpstra-Jonckbeere Trend Test, showed a significant increase in % days spent in estrus ($p<0.01$). At 12 months there was also a dose-related trend ($p<0.05$) but the increases in dosed groups compared to controls as determined by ANOVA were not significant at either dose.

The percent days in proestrus is similar in dosed animals compared to controls for all timepoints. There is a significant trend ($p<0.01$) in both dose groups for a decrease in % days spent in proestrus over time. This decrease was seen in controls also and is to be expected. Atrazine exposure did not appear to effect this trend.

The percent days spent in diestrus was significantly decreased in dose groups compared to controls at nine, 12 and 18 months. Females at nine months spent a mean of 44.8% of the days of the estrous cycle in diestrus. Dosed females spent 36.2% ($p<0.05$ compared to control) for the 70 ppm dose and 25.9% ($p<0.01$ compared to control) of the days in diestrus. The dose-related trend at nine months was significant at $p<0.01$. At 12 months there was also a dose-related trend that was significant at $p<0.01$. The 4.23 mg/kg/day group, at 12 months, was decreased 14% compared to control, but this was not statistically-significant. The 24.23 mg/kg/day group at 12 months was decreased 28% compared to controls and this was significant ($p<0.05$). The trend at 18 months was decreased in a dose-related manner ($p<0.01$) while both the 4.23 and 24.23 mg/kg/day groups compared to controls by ANOVA were also statistically-significantly decreased ($p<0.05$, for both).

To summarize the estrous cycle data from this study:

-- When analyzed for effect over time it was seen that animals in both dose groups and the controls exhibited an increase in % days in estrus and a decrease in % days in di- and proestrus as the study progressed. These alterations are to be expected in an aging SD female rat and exposure to atrazine did not appear to affect these parameters compared to controls.

-- When analyzed for effect of dose it was seen that there was a significant increase, compared to controls, in percent days spent in estrus at nine, 12 and 18 months in both dose groups. The percent days spent in diestrus significantly decreased in both dose groups compared to controls.

9.2.3.5 Results of Estrous Cycle Measurements in the SD Rat (Morseth, 1996a and 1996b)

These studies examined the effect of atrazine on the estrous cycle and on plasma concentrations of the hormones LH and prolactin. The hormone measurement data from these studies will be discussed below. Preovulatory LH levels, and the estrous cycle evaluations will be discussed here. The studies were of one month (Morseth, 1996a) and six months (Morseth, 1996b) in duration.

One-Month Study. Female SD rats were exposed to 0, 2.5, 5, 40 and 200 mg/kg/day technical grade atrazine for 28 to 31 days. Ninety females per dose group were used. Dosing was by gavage once a day, at approximately the same time each day.

The results of the smears indicated an effect of atrazine exposure on vaginal cycling. Atrazine exposure induced a dose-dependent increase in the number of animals having irregular cycles. The nature of the irregular cycles was both an increase in estrous cycle blocks (two consecutive days in estrus) and an increase in diestrus blocks (four consecutive days in diestrus). The effects of atrazine on the estrous cycle in this study are most pronounced at 40 and 200 mg/kg/day with a statistically-significant increase in females displaying diestrus blocks at both 40 and 200 mg/kg/day and a statistically-significant increase in females displaying estrus blocks at 200 mg/kg/day only (p 0.05 for both diestrus and estrus blocks using pairwise comparison). These data are shown in Appendix Table 9.

Six-Month Study. Female SD rats were exposed, through the diet, to 0, 1.8, 3.65 and 29.4 mg/kg/day technical grade atrazine for six months. Ninety females per dose group were used.

A statistically-significant increase in percent days in estrus was evident as early as 3.5 months into the study in the high dose group, and in the mid-dose group by 5.5 months into the study. The low-dose group never experienced statistically-significant alterations in their estrus cycles. These data are shown in Appendix Table 10.

9.2.3.6 Results of Estrous Cycle Measurements in the SD Rat (Morseth, 1998)

The estrous cycle evaluations performed in this two-year bioassay demonstrate that atrazine-treated females display increased days in estrus sooner than control animals. All animals, irrespective of dose group, spent a normal amount of time in estrus (approximately 25% of the days spent in estrus) for the first 10 weeks of the study. By the 13 to 14 week measurement period though, the atrazine-treated animals began to display more days spent in estrus. These increases were most evident in animals of the high-dose group while, for most timepoints, the other three dose groups showed only marginal increases in percent days in estrus. The differences between the control and dose groups were most evident at week 25 to 26 where controls spent 53.7% of the days in estrus compared to 63.8, 59.7, 55.4, and 72.1% of the days in estrus for 1.5, 3.1, 4.2, and 24.4 mg/kg/day groups. Appendix Table 11 displays the percent days in estrus by dose group for all the time periods up to 46 weeks. Animals dosed with atrazine also showed an increase in the likelihood of having an estrus block of seven days during one of the three measurement periods in the 17-26 week interval. Again, this effect was most evident at the high-dose group, but was also seen at the other dose groups. Appendix Table 12 displays these data.

In addition to examining the amount of time spent in estrus by atrazine-treated animals compared to control animals, this study also examined the relationship between amount of time spent in estrus and mammary tumor incidence. There was a clear relationship between the amount of time spent in estrus and mammary tumor onset. These data are shown in Appendix Tables 13 and 14.

9.2.3.7 Results of Ovarian Histomorphology and Estrous Cycle Measurements in the F-344 Rat (McConnell, 1995)

The only study that performed histomorphologic examination of the ovaries of atrazine-treated Fischer rats was the Thakur, 1991b study. The histomorphologic evaluation data is presented as McConnell, 1995 while the vaginal smear data (for determination of phase in estrous cycle) is presented as Eldridge, 1993b. HED believes that the estrous cycle evaluation (vaginal smears) reported in Eldridge, 1993b are unreliable. Thus, these data are not reported. The histomorphology data from McConnell, 1995 are instead used to determine stage of the estrous cycle.

Ovarian Histomorphologic Examination in the F-344 (McConnell, 1995). The great majority of F-344 rats in all groups, both control and dose groups, maintained CL throughout most of the study. Only at the final, 24-month, timepoint were there dramatic decreases in CL numbers. The atrazine-treated animals at this timepoint did not show decreases in CL numbers any more severe than the control animals. The reduction in CL numbers at this late timepoint appears to be a consequence of a natural progression of the animals from persistent diestrus into acyclicity. All animals in all dose groups maintained moderate numbers of secondary, antral and atretic follicles throughout the study - including the 24-month timepoint.

Histomorphology to Determine Stage of the Estrous Cycle in the F-344 (McConnell, 1995). All animals in all dose groups appeared to maintain normal cycles through the first 12 months of the study. At the 15-month timepoint approximately half the animals in all dose groups (five of ten in control; three of ten at 0.68 mg/kg/day; 6 of 11 at 4.82 mg/kg/day; five of ten at 14.05 mg/kg/day, and five of ten at 34.33 mg/kg/day) were in a state of extended diestrus. The state of extended diestrus was indicated by the presence of increased vaginal mucification -- which is indicative of extended diestrus (McConnell, 1989a). At the 18-month timepoint approximately 70 to 80% of the animals in all dose groups displayed increased vaginal mucification. The persistent diestrus (or pseudopregnancy) that is characteristic in an aging F-344 was evident by 15 months and was quite common by 18 months. At 24 months the incidence of vaginal mucification was still high, but the previously mentioned reduction in animals with CL indicated that a progression towards acyclicity was occurring in the animals in this study.

9.2.4 Summary And Discussion From The Ovarian Histomorphology and Estrous Cycle Measurements In F-344 and SD Strains

Over the duration of the study, both strains of rat exhibited ovarian histomorphology and estrous cycles that would be expected for those strains. The F-344 maintained CL throughout most the 24-month study and showed an increase in days spent in diestrus (as indicated by vaginal mucification) at the post-12 month timepoints (McConnell, 1995). The SD showed decreased numbers (and frequently, a complete absence of) CL, increases in secondary, antral and atretic follicles, and an increase in percentage of days in estrus (as indicated by the vaginal smears) as the study progressed (Eldridge, 1993b; McConnell, 1995).

Atrazine exposure in the F-344 did not seem to alter estrous cycles; atrazine exposure in the SD increased the number of estrus and diestrus blocks after as little as one month of exposure (Morseth, 1996a). Three months of exposure increased the percentage of days spent in estrus and decreased the percentage of days spent in diestrus, but not significantly so (Thakur, 1991a). By nine months of atrazine exposure the percentage of days spent in estrus were significantly increased compared to controls and the percentage of days spent in diestrus was significantly decreased. The increase in estrus and diestrus blocks after one month of atrazine exposure indicates that these females were cycling irregularly. The increased days spent in estrus at three and nine months indicates that the atrazine-exposed animals in this study were entering constant estrus sooner than the control animals.

As noted above, reproductive aging is manifested in the estrous cycle first as irregular cycling, then as constant estrus, and finally as acyclicity/persistent diestrus. Atrazine exposure was able to induce irregular cycling in the SD females after only one month of exposure - when the animals in this study were about three months of age.

The Thakur, 1991a; Eldridge, 1993a, and the two shorter duration Morseth studies indicate that atrazine exposure can alter estrous cyclicity in the female SD rat. Atrazine exposure did not seem to effect estrous cyclicity in the F-344 rat (McConnell, 1995).

The estrous cycle data from the Morseth two-year study (described in Thakur, 1999) confirmed the findings from Eldridge (1993a) and the shorter duration Morseth studies (Morseth 1996a and 1996b). In this study atrazine exposure resulted in SD females spending an increased amount of time in estrus earlier than control animals. The high-dose group (26.23 mg/kg/day) females in the Thakur study showed a group mean of 44.8% of the days in estrus at nine months. The control animals reached this level of days in estrus, but not until 12 months when their group mean days in estrus was 42.6%. The high-dose (24.4 mg/kg/day) females in the Morseth, 1998 study had a group mean of 47.8% of the days in estrus during weeks 17 to 18. The control animals reached this level of days in estrus, but not until weeks 21 to 22 when their group mean days in estrus was 45.6%. All four studies together provide strong evidence that atrazine can disrupt estrous cycles in the SD female and can lead to an early onset of increased percent days in estrus compared to control animals.

The Morseth (1998) study examines correlations between days in estrus and mammary tumor incidence and onset and demonstrates that increased days in estrus early in the life of an animal decreases the time to onset of mammary tumors. This Morseth study also demonstrated that even animals who are not spending increased time in estrus during the early period of life, will show an increased risk of mammary cancer when chronically fed atrazine.

9.2.5 Serum Estradiol and Prolactin Levels

Given the postulated mode of action, it is important that one examine serum estradiol and prolactin levels and confirm that they are, in fact, altered following exposure to atrazine.

Serum hormone levels of estradiol (as well as progesterone, prolactin and corticosterone) in atrazine exposed SD female rats were examined in Eldridge, 1993a. Serum hormone levels of these four hormones were examined in F-344 females in Eldridge, 1993b.

Serum corticosterone was measured as an indicator of stress while estradiol, prolactin, and progesterone were measured because of their roles (estrogen and prolactin as promoters of and progesterone as a possible inhibitor of) mammary carcinogenesis.

Eldridge 1993a and 1993b are discussed below. The histomorphological evaluation of the mammary gland in atrazine-exposed SD rats is also discussed below (McConnell, 1995)

9.2.5.1 Protocol and Rationale for Hormone Measurement

Doses and sacrifice schedules for these two studies were previously described. The protocol and measurements for hormone levels in these studies are described below.

Blood was collected from the trunk of all animals at scheduled sacrifice. Two weeks prior to each scheduled sacrifice vaginal smears were performed. Animals were sacrificed during proestrus, if possible. If, after 21 days of vaginal smears, an animal was not in proestrus, then this animal was sacrificed, irrespective of what stage of the estrous cycle the animal was in. Blood samples were used for the determination of serum estradiol, progesterone, prolactin, and corticosterone. The hormones estradiol, progesterone, and prolactin all are important in the regulation and maintenance of normal reproductive functioning in the rat and could potentially play roles in mammary tumor pathogenesis. Corticosterone measurements were taken to test the hypothesis that dosing the animals with atrazine produces stress (and thus an elevation of serum corticosterone levels) that may play a role in mammary gland neoplasia.

Radioimmunoassay techniques were used to measure levels of each hormone. Standard curves were constructed and sample values were compared to the standard curve. The results of the measurements were examined for alterations in:

- each individual timepoint compared to controls;
- trend within dose, and;
- effect of treatment over time.

9.2.5.2 Results of Hormone Measurements -- F-344 (Eldridge, *et al*., 1993b)

The results of the hormone measurements did not reveal any consistently statistically-significant alterations in serum hormone levels compared to controls for any of the hormones tested. There were occasional significant alterations such as significantly decreased ($p < 0.05$) progesterone levels in the 4.82 mg/kg/day dose and corticosterone levels in the 34.33 mg/kg/day group at the 12-month timepoint compared to controls; significant negative trends in estradiol levels at the 12-month timepoint, progesterone levels at the 12- and 18-month timepoints, and corticosterone levels at the 12- and 15-month timepoints, and prolactin levels displayed a significant positive trend at the three-month timepoint only. Careful consideration of these alterations indicated that they did not appear to be related to atrazine exposure.

There were alterations in serum hormone levels that were seen in control as well as treated rats. These were: decreases in estradiol levels in the later half of the study; significantly increasing progesterone levels from one to18 months. Decreased estradiol and increased progesterone are expected in rats undergoing a reproductive aging process involving pseudopregnancy (Huang *et al.,* 1978). Exposure to atrazine did not alter the age-related changes in estradiol or progesterone levels.

An increase in prolactin levels might be expected in an

aging rat undergoing pseudopregnancy. However, consistent increases in serum prolactin levels were not seen.

9.2.5.3 Results of Hormone Measurements - SD (Eldridge *et al.*, 1993a)

Appendix Table 15 displays the results of the hormone measurements in the SD females.

Serum progesterone and corticosterone levels did not show any significant dose-related alterations compared to controls. Serum prolactin levels in dosed groups did not show any significant dose-related alterations with the exception of the 26.23 mg/kg/day group that did however, show a negative trend ($p<0.01$) over the nine to 18 month period and a positive trend at nine months. These alterations in serum prolactin likely were related to compound exposure.

Serum estradiol levels in the control rats in this study showed a positive trend (levels increased as time increased) over months one through nine. Exposure to atrazine did not alter this trend. The 4.23 and 26.23 mg/kg/day dose groups also showed a significantly positive trend from months one through nine. These trends are expected as constant estrus would be expected to begin to set in as these animals approach nine months of age. Examination of the pairwise comparisons at three months indicates that treated animals had an early onset of increased serum estradiol levels compared to controls. At three months control estradiol levels were 3.5 ng/mL, 70 ppm levels were 11.2, and 400 ppm levels were 16.2 ng/mL. The increase at 70 ppm was significant at $p<0.05$, the increase at 400 ppm was significant at $p<0.01$ and the trend, determined using a Terpstra-Jonckbeere Trend Test, was positive at $p<0.05$. At nine months control and 4.23 mg/kg/day group estradiol levels were similar, but the 26.23 mg/kg/day group compared to controls was increased 44%. At nine months estradiol levels were elevated compared to control, but not significantly so.

Prolactin levels were not altered either as a result of atrazine exposure or as result of aging in this study.

9.2.5.4 Results of the Histomorphologic Evaluation of the Mammary Gland - SD (McConnell, 1995)

The mammary gland is clearly a hormone-responsive tissue. Various tissues in the mammary gland contain receptors for the hormones estrogen, progesterone and prolactin and exposure to these hormones effects on these tissues. A detailed histomorphologic analysis of mammary gland (and other) tissues from the rats in the Thakur, 1991a study was performed. This histomorphologic analysis is referred to in this document as McConnell, 1995.

The mammary glands were examined for these alterations:

- Acinar development - indicative primarily of estradiol exposure
- Acinar/lobular development- indicative of both prolactin and progesterone exposure
- Secretory activity - indicative of prolactin, and to a lesser extent, estrogen and progesterone exposure
- Dilated ducts with secretion - indicative of prolactin, and to a lesser extent, progesterone exposure
- Galactocele (milk cyst) - indicative of prolactin, and to a lesser extent, progesterone exposure

The alterations in the tissues of the acinar region are indicative of estrogen exposure (ductal epithelial hyperplasia and acinar development). The alterations related to milk production are primarily prolactin-dependant (secretory activity, dilated ducts with secretion, galactocele).

Appendix Table 16 displays the results of the histomorphologic analysis of the mammary glands at months one, three, nine and 12 in the SD rats from Thakur, 1991a. Values at 15, 18 and 24 months are not shown as these values are similar to control values. The index weighted scores of several of the above listed parameters are shown in this table. An index weighted score assigns a numerical value to the severity of the finding assigned by the pathologist. The higher the index weighted score, the more severe was the finding in that group.

Increased prolactin exposure in the rat is associated with formation of galactoceles (milk cysts). Galactocele incidence and severity in this study are shown in Appendix Table17. The results of the histomorphologic analysis are described below:

Acinar Development. An early onset of increased exposure to estrogen is indicated by examination of the column of Appendix Table 16 labeled "Acinar Development." The index scores at the one-month timepoint are slightly higher in the dose groups than in the control, but a dose-response relationship was not seen. At the three-month timepoint the index scores are again increased over control. The increase is at this timepoint is dose-related though with the high dose being more severe than the low dose. At both nine and 12 months the index score again indicate more severe acinar development in the dose groups compared to the controls with the increase in severity being especially obvious at the high dose.

Acinar/Lobular Development. An early onset of this parameter is evident. The one- and three-month timepoints have index scores in the dose groups that are similar to the control index scores. The dose group index scores are clearly increased compared to controls at nine and 12 month timepoints though. This indicates that the atrazine-treated animals were exposed to elevated levels of prolactin at an earlier timepoint than the control animals.

Secretory Activity. The index scores for secretory activity also demonstrate an early onset of increased prolactin exposure in the dose groups compared to the controls. Index scores at one and three months in the dose groups were similar to control values, while index scores in both dose groups were clearly elevated compared to controls at the nine- and 12-month timepoints.

Dilated Ducts with Secretion. Index scores at one and three months in the dose groups were similar to control values. The index scores for the 4.23 mg/kg/day group compared to controls were only slightly elevated. The index scores for the 26.23 mg/kg/day group were greatly elevated compared to controls

Galactocele Incidence and Severity. No galactoceles were observed in any group at the one- and three-month timepoints. At nine and 12 months galactoceles in the dose groups were increased in both number and severity. By 15 months galactocele incidence and severity were similar between control and dose groups.

9.2.6 Summary And Discussion Of The Hormone Measurements and Histomorphologic Alterations In F-344 And SD Strains

Serum corticosterone levels were not altered in F-344 or SD female rats by atrazine exposure. Serum corticosterone levels reflect stress and the lack of any alteration in these levels indicates that the dosing, and more importantly, the regular vaginal lavages, were not causing the animals in these studies undue stress that may have compromised the results of these studies.

Atrazine exposure in the F-344 females did not alter serum hormone levels of estradiol, progesterone or prolactin. Both control and dose groups did see decreases in serum estradiol levels for the latter half of the study and a generally increasing level of progesterone for the one to 18 month period. These changes in estradiol and progesterone are to be expected in animals undergoing reproductive aging through pseudopregnancy. What was not seen, but would be expected in an animal undergoing reproductive aging through pseudopregnancy, were increases in serum prolactin levels.

Accurate serum prolactin levels from the rat can be difficult to obtain because many different factors can cause dramatic alterations in serum prolactin levels. For example, simple inadvertent stimulation of a female rats nipples can induce large increases in serum prolactin (Freeman, 1981). Prolactin levels are also very sensitive to stress. A rat in pseudopregnancy is especially difficult to obtain accurate serum prolactin measurements from as these animals will display twice-daily prolactin surges. Prolactin measurements from these animals will vary dramatically depending on whether or not a measurement is taken during a surge, and, if taken during a surge, at what point in the surge. Accurate prolactin measurements in a young, unmated rat, are easier to obtain as these animals have relatively static prolactin levels except for have one, or possibly two, prolactin surges every four days (a proestrus afternoon surge and, sometimes, a smaller surge on estrus) (Freeman, 1981; Butcher *et al.,* 1974).

Despite these difficulties, it is still surprising that increased prolactin levels were not measured in this study in the aging animals.

Atrazine exposure in the SD females did not alter serum progesterone levels or serum prolactin levels in dose groups compared to controls. The serum estradiol levels in the SD rat were dramatically altered by atrazine exposure and deserve special attention. Atrazine exposure resulted in an early exposure to high levels of estrogen. The levels of serum estrogen in the dosed groups of 70 and 400 ppm females at three months were 11.2 ± 12.6 and 16.2± 13 ng/mL compared to only 3.5 ± 6.4 ng/mL in the control females at this time point. While the standard deviations in these groups are large the increases are three to over four-fold, and the increases are statistically-significant using a pairwise comparison. The atrazine exposed groups had higher serum estrogen levels than controls at the three-month timepoint. There was also a positive dose-related trend over the first nine months of the study in estradiol levels. Exposure to such high levels of estrogen this early in the rats life is not normal. Exposure to these levels of estrogen at nine months, as can be seen from Appendix Table 15, is normal. The early exposure to these high estrogen levels may be leading to an earlier onset of mammary tumors.

As was the case with the F-344, increased prolactin levels would be expected as a consequence of the normal aging process in the SD; yet increases in serum prolactin were not seen in this study. Old SD females with pituitary adenomas can have serum prolactin levels that are approximately 13 times higher than in young SD females (Sarkar *et al.*, 1982). An increase in all the groups (both control and atrazine-treated) would be expected as the pituitaries increased in size and pituitary adenomas became common. By the 18- and 24-month timepoints the majority of the animals in all groups had pituitary adenomas; yet serum prolactin levels at these two timepoints were similar to control values. Increases in serum prolactin levels have been seen in numerous studies in the published literature and are accepted to be a normal part of the aging process in the SD female and other rats that undergo constant estrus as the predominant mode of reproductive aging (Cónsole *et al.*, 1997; McComb *et al.*, 1984; Sandusky *et al.*, 1988; Baird *et al.*, 1990; McComb *et al.*, 1986; van Putten *et al.,* 1988).

The fact that measured serum prolactin levels were not increased with age in either strain must be considered a weakness in this study, despite the difficulties of such measurements. This is especially true in the SD study where very large increases in serum prolactin levels should have been evident.

Because of the lack of increased serum prolactin (as determined by direct serum measurements) in the aged animals in Eldridge, 1993a, the histomorphologic data from this study was examined especially closely for any signs of increased prolactin exposure.

The incidences and severity of all four parameters that indicate increased exposure to prolactin (acinar/lobular development, secretory activity, dilated ducts with secretion, galactoceles) were increased in atrazine-treated groups compared to controls at the nine and 12-month timepoints. Index scores for values before nine months and after 12 months were similar among dose groups. The increased index scores at nine and 12 months in dose groups compared to controls indicates an early onset of increased prolactin exposure in the dose groups compared to the control. With time, the normal aging process proceeds in the control animals and by 15 months the control animals have "equalized" or "caught up" with the dose groups. Thus, index scores are similar for the timepoints after 12 months. Index scores are similar for the timepoints prior to nine months because increases in prolactin require first that estrogen be increased. The increased estrogen then acts at the pituitary to induce lactotroph hyperplasia that results in increased prolactin levels and, finally, increased incidence and severity of these mammary gland findings. Appendix Table 15 shows increases in serum estradiol at three months in the dose groups compared to controls while Appendix table 16 shows a dose-related increase in acinar development (primarily an estrogen-dependent effect) at three months in the dose groups compared to control groups. The increased serum estrogen levels seen at three months are affecting the pituitary between three and by nine months the pituitary lactotrophs are proliferating and producing prolactin such that by nine months prolactin-dependent alterations at the mammary gland are evident in the dosed animals.

9.2.7 Preovulatory LH Levels

The effect of aging on the preovulatory luteinizing hormone (LH) surge, has been briefly discussed previously in this document. As in humans, ovulation is triggered in rats by a sudden and dramatic increase (a surge) in serum LH levels. The attenuation of the preovulatory LH surge in aging female rat strains, including the SD, has been well-described (Lu *et al.*, 1979, Cooper *et al.*, 1980). One- and six-month studies examining the effect of atrazine exposure on the preovulatory LH surge are available (Morseth, 1996a; Morseth, 1996b; Minnema, 2000). These data show that atrazine exposures as short as one month can dramatically attenuate the pre ovulatory LH surge. Plasma prolactin levels were also determined in the Morseth, 1996a study and results of this analysis were reported. Plasma prolactin concentrations have been shown to undergo a preovulatory surge in rats similar to the LH surge (Butcher *et al.,* 1974). However, this document will not go into a detailed discussion of the effect of atrazine on the plasma prolactin surge simply because the role of this event in female rat inducing ovulation are not as well described as a the LH surge.

9.2.7.1 Protocol for LH Surge Measurement -- One-Month Study (Morseth, 1996a)

Female SD rats were exposed to 0, 2.5, 5, 40 and 200 mg/kg/day technical grade atrazine for 28 to 31 days. Ninety females per dose group were used. Dosing was by gavage once a day, at approximately the same time each day. After 28 to 31 days of atrazine exposure the animals were OVX. Vaginal smears were performed from days seven to the day prior to OVX to determine the animals cycling patterns. Estradiol implants were placed in each animal seven days following OVX and the animals were sacrificed three days later. Thus, OVX occurred 10 days prior to sacrifice and estradiol implants occurred three days prior to sacrifice. This protocol of OVX followed by estradiol implantation, followed by sacrifice has been previously used by other investigators to induce an LH surge in SD female rats (Legan *et al.*, 1975).

Ovarectomization of the animals followed by implantation of

estradiol implants was done in an attempt to synchronize the estrous cycles of the animals so that the LH surge in each animal would occur at approximately the same time. This allows for comparison of the surge between dose groups and also points the investigator to a specific time at which the LH surge should be occurring. With this information the animals may be bled and serum LH levels measured. Animals were bled for serum hormone measurements at six different timepoints spread over 12 hours. The first two time points were 1100 and 1400 in biologic time (biologic time being time in the light cycle - biologic time 1200 is mid-point of the light cycle or noon of the light cycle). These first two timepoints are baseline. The other timepoints for serum measurement are biologic time 1600, 1800, 2000, and 2300. The peak of the LH surge would be expected to occur in the late afternoon of the light cycle (around 1800 biologic time). This equates to the late afternoon of proestrus in a normally cycling rat. By 2300 biologic time the LH surge would be expected to be over and LH values should return to baseline levels.

Out of the 90 animals in each group 10 were "repeat bleed." These animals were bled at each timepoint from the jugular vein for the first four bleedings, through the ocular venous plexus for the fifth bleed and the trunk for the last timepoint. The remaining 80 animals in each dose group were sacrificed, and trunk blood was collected according to the schedule shown in Appendix Table 18.

9.2.7.2 Results of LH Surge Measurements -- One-Month Study

The means and standard deviations for serum LH measurements from this study are shown in Appendix Table 19.

Non-repeat Bleed. Plasma LH values of the 200 mg/kg/group were significantly decreased at 1600 and 1800 compared to controls at that timepoint (specific p value not given). There were also non-significant decreases in plasma LH levels, compared to controls, in the 5 and 40 mg/kg group (45.4% and 36.8%, respectively). There was not a great increase in the 200 mg/kg group in the magnitude of the peak response over its own baseline value. Control mean baseline (1100 and 1400 hours) values are 998 and 1122 pg/mL compared to a peak value of 5138 pg/mL at 1800: approximately a five -fold increase. The baseline values for the 200 mg/kg group are similar to controls - 873 and 1099 pg/mL. However, the peak value in the 200 mg/kg group is only 2752 pg/mL: an increase of only 2.5-fold.

Repeat Bleed. Peak values compared to controls in the 200 mg/kg group were, as in the non-repeat bleed set, significantly decreased. The peak control value (1800 hours) was 2650 pg/mL while the peak 200 mg/kg value was 812 pg/mL (1800 hours). The 40 mg/kg group was decreased compared to controls (1450 pg/mL) but not significantly so. There was little increase in the 200 mg/kg group in the magnitude of the response over its own baseline value. Control baseline values were 732 and 786 pg/mL compared to peak values of 2650 pg/mL: approximately a 3.5-fold increase. Peak values in the 200 mg/kg group were increased only about 45% over baseline values (812 vs. 514 and 453 pg/mL).

9.2.7.3 Summary of the Plasma LH Measurements from the One-Month Study

The plasma LH values seen in the control animals in both the repeat and non-repeat bleed data indicate that one month of atrazine exposure dramatically attenuates the preovulatory LH surge in the high-dose group of 200 mg/kg/day. This effect is only statistically-significant in the high-dose group of 200 mg/kg, but it can be seen in the 40 mg/kg/day group also.

The results seen in this study are confirmed by a separate 28-day using similar protocols and identical doses (Minnema, 2000). The LH surge, in this study, was statistically significantly attenuated at both the 40 and 200 mg/kg/day doses.

9.2.7.4 Protocol for LH Surge Measurement -- Six-Month Study (Morseth, 1996b[4])

The protocol for this study was very similar to the protocol for the one-month study. The main differences were:

- duration of atrazine exposure (26 weeks);
- route of exposure (through the diet);
- and, dose levels (25, 50 and 400 ppm - 1.8, 3.65, and 29.44 mg/kg/day).

Other than these differences, the six-month study was conducted much the same as the one-month study. Animals were OVX 10 days prior to sacrifice and implanted with estradiol implants three days prior to sacrifice. Ninety females per group were used in a sacrifice schedule identical to that used in the one-month study (shown in Appendix Table 18). Blood collection and plasma hormone measurements were performed identical to the methods used in the one-month study.

[4]Data from the study referred to here as Morseth 1996b has been published in the open literature as Eldridge *et al*., 1999.

9.2.7.5 Results of LH Surge Measurements -- Six-Month Study

The means and standard deviations for serum LH measurements from this study are shown in Appendix Table 20.

Non-repeat Bleed. Plasma LH values of the 400 ppm group were significantly decreased at 1400, 1800, and 2000 hours compared to controls at those timepoints (specific p value not given). There was not a large increase in the 200 mg/kg group in the magnitude of the response over its own baseline value (specific p value not given). Control mean baseline (1100 and 1400 hours) values are 1900 and 2326 pg/mL compared to a peak value of 3458 pg/mL at 1800: approximately a 1.6 -fold increase. The baseline values for the 200 mg/kg group are slightly less than controls - 1863 and 1420 pg/mL. However, the values at 1600 and 1800 in the 200 mg/kg group are only 1913 and 1356 pg/mL. The average of the 1100 and 1440 hour and 1600 and 1800 hour in the 200 mg/kg groups are essentially the same - 1641 and 1634 pg/mL.

Appendix Table 20 displays the baseline values, peak LH values and % increase of peak values over baseline. Examination of this table shows that at the high-dose group of 29.4 mg/kg/day there is clearly a decrease in the strength of the LH surge. At this dose level the surge does not seem to be occurring at all.

Repeat Bleed. Baseline values for plasma LH are similar among controls and all dose groups. There is a statistically-significant decrease at 1600, 1800, 2000, and 2300 in the 29.4 mg/kg/group compared to controls. The 50 ppm (3.65 mg/kg/day) group had a decrease at 1800 (25%) compared to controls. Compared to its own baseline values, the LH values in the 29.4 mg/kg group were not altered. Values at 1600 and 1800 are actually slightly lower than baseline values. Appendix Table 20 displays the baseline values, peak LH values and % increase of peak values over baseline. Appendix Figure 1 displays a line graph of the results from the repeat bleed group of this study.

9.2.7.6 Summary of the Plasma LH Measurements from the Six-Month Study

An attenuation of the LH surge at the high dose of 400 ppm (29.4 mg/kg/day) is clear. Examination of the data from Appendix Table 20 shows that plasma LH values for both the repeat and non-repeat bleeds at this dose remain essentially flat over the six timepoints.

The other dose groups do not appear to be as affected by atrazine exposure. The non-repeat bleed data for the dose groups is very similar to controls. There is a decrease in the strength of the LH surge at the mid-dose of 50 ppm (3.65 mg/kg/day), but the magnitude of this decrease is not large and given the variability inherent in this assay (as indicated by the large standard deviations), it is difficult to draw firm conclusions based on this decrease.

9.2.8 Summary And Discussion Of The LH Surge Studies

In the non-repeat bleed set of the one-month study the baseline values for the 200 mg/kg/day group are similar to controls. However, the peak value in the 200 mg/kg/day group is increased only 2.5 fold -- much less than the five-fold increase seen in control peak values versus control baseline values. The results from the repeat bleed set for the one-month study are even more indicative of an attenuated LH surge. Baseline values for both control and 200 mg/kg/day groups are, again, similar. The peak LH values are much less in the 200 mg/kg group compared to the control though. Peak control LH values in the repeat bleed set were increased approximately 3.5-fold over baseline. Peak values in the 200 mg/kg group were increased only about 45% over baseline values.

The results seen the six-month study in the 29.4 mg/kg/day group also indicate an attenuated LH surge. Both the repeat and non-repeat data sets show plasma LH levels that are flat over time. Control LH values at their peak are about 67% above baseline values for the non repeat bleed data set and about 226% above baseline values in the repeat bleed set. Peak values in the 29.4 mg/kg group are 17% (non-repeat bleed) and 20% (repeat bleed) lower than baseline values though.

A major factor to consider when drawing conclusions from this study is, as indicated above, the large standard deviations in the data. Examination of the data shown in Tables 20 and 21 shows that the standard deviations are quite large and, in fact, sometimes exceed the means. An extreme amount of variability is to be expected with this type of data. Variability can be expected between rats in both timing and magnitude of the LH surge. Although the animals were synchronized by photoperiod and by OVX, there will still be variability in timing of the surge among rats in a dose group. It is hoped that all the rats will have their plasma LH levels be at their peak at 1800 hours when they are sacrificed and blood is collected. Clearly, however, this will not be the case. Some rats will experience a peak LH surge prior to 1800 hours and some after. The magnitude of the LH surge peak will also vary among rats. This is due largely to the differential rate at which the animals reproductive systems age. The variability of reproductive aging among female rats in a particular strain has been well described (Cooper *et al.*, 1986; Lu *et al.*, 1994; LeFevere and McClintock, 1988). Because the LH surge is attenuated as part of the female SD rats reproductive aging process, the variability in rate of reproductive aging means that animals of the same chronological age will have LH surges of varying magnitude.

Even given the variability inherent in the LH measurements in this type of study, there are some conclusions that can be reached with confidence. There is little doubt that in the one-month study at 200 mg/kg/day and in the six-month study at 29.4 mg/kg/day, there is an attenuation of the LH surge. Appendix Figure 1 displays a line graph of the mean plasma LH levels in the repeat bleed group from the six-month study displayed without standard deviation or standard error bars. The decreased LH surge at 3.65 mg/kg/day in the six-month study is less apparent but may be considered biologically-significant.

9.3 The Site of Action for Atrazine Attenuation of the LH Surge

Experiments have been conducted examining the mechanisms underlying the attenuation of the LH surge produced by atrazine. The focus of these experiments has been the target site of action in the brain for atrazine. The LH for the proestrus afternoon LH surge comes from the anterior pituitary and the release of LH from the pituitary is controlled by gonadotropin releasing hormone (GnRH), which is produced in the hypothalamus. Thus, atrazine could be altering the LH surge by acting at either the hypothalamus (and affecting GnRH - the signal to release LH) or by acting at the pituitary and directly affecting its ability to secrete LH.

Experiments examining the effects of atrazine exposure on the hypothalamus and pituitary were conducted at the Reproductive Toxicology Division of National Health and Environmental Effects Research Laboratories (Cooper *et al.*, 1998, Cooper *et al.*, 2000, Das *et al.*, 1999, Das *et al.*, submitted). These studies, through the results of both *in vivo* and *in vitro* experiments, demonstrate that atrazine appears to be attenuating the LH surge by acting on the hypothalamus, rather than directly affecting the pituitary.

An *in vivo* experiment using the Long-Evans (LE) strain of rat, administered GnRH through a cardiac catheter to OVX atrazine-treated females to see if GnRH exposure could reverse the atrazine-induced attenuation of the LH surge. Females, in this study, given atrazine only showed an attenuation of the LH surge -- which was expected. Females given atrazine plus 50 ng/rat of GnRH, did not display an attenuated LH surge. This provides evidence that atrazine is affecting the ability of the hypothalamus to release GnRH.

An *in vitro* experiment using perfused anterior pituitaries removed from untreated female LE, showed that atrazine could not directly affect the ability of the anterior pituitary to secrete LH. Pituitaries perfused *in vitro* were able to produce an LH surge when primed with estradiol. Adding 100 µM atrazine to the perfusion system did not affect the ability of the pituitaries to produce an LH surge following estradiol priming.

These two experiments taken together provide evidence that atrazine inhibits the proestrus afternoon LH surge through an action on the hypothalamus rather than a direct action on the anterior pituitary. Specifically, it appears that atrazine may somehow inhibit the hypothalamic secretion of GnRH.

Data from the Cooper lab indicates that a decrease in hypothalamic norepinephrine levels may be responsible for the reduced capacity of the hypothalamus to secrete GnRH (Cooper *et al.,* 1999a). In these studies, exposure of LE rats to a three-day exposure of 50, 100, 200 and 300 mg/mL/day of atrazine resulted in significant depressions of hypothalamic norepinephrine levels at all dose levels. This study is supported by *in vitro* studies using triazines and PC12 cells that showed that exposure through the medium of 50, 100 and 200 µM atrazine resulted in dramatic decreases in norepinephrine release at all dose levels starting as early as six hours following the start of exposure and continuing for up to 48 hours following exposure (Das, *et al. ,* 1999).

Disruption of GABAergic neurotransmission by atrazine may also play a role in the decrease in GnRH release seen following atrazine exposure. Gamma-aminobutyric acid type a receptors ($GABA_A$) are known to play a crucial role in GnRH release. *In vitro*, atrazine (and cyanazine) have been shown to disrupt agonist binding to the $GABA_A$ receptor in cortex from male Long-Evans rats (Schafer, *et al.*, 1999). Such disruption could contribute to the decreased hypothalmic GnRH release seen following atrazine exposure.

9.4 The Data Examining the Association Between Atrazine Exposure and An Attenuated Proestrus Afternoon LH Surge, Increased Days and Estrus and a Prolonged Exposure to an Elevated Level of Estradiol

This document presents several studies examining an association between atrazine exposure and an early onset of alterations in the above described parameters - estrous cycle, serum estradiol levels; serum LH levels. A study is also available examining the correlation between estrous cycles and mammary tumor incidence/onset. The time of tumor onset is also examined in several studies in an attempt to confirm that normally occurring events (mammary tumor induction) are, in fact, occurring earlier following atrazine exposure.

The results indicate that atrazine exposure does appear to result in an early onset of these parameters. Increased days in estrus, increased serum estradiol levels, attenuated LH surge, and onset of mammary tumors all occur earlier in atrazine-treated females than they do in untreated females.

9.4.1 Atrazine Exposure Results in an Earlier Onset of Increased Days in Estrus

One-month of atrazine exposure at 40 mg/kg/day induced estrus blocks. Longer term studies revealed that atrazine exposure at lower levels also has the ability to increase days in estrus if the exposure is of long enough duration. A dose of 3.65 mg/kg/day in a six-month study was able to induce an increase in days spent in estrus over controls as early as 5.5 months into the study. A dose of 4.23 mg/kg/day in a two-year study (the low-dose tested in this particular study) induced an increased percentage of days spent in estrus at nine months -- an event that is not seen in control SD females in this study until about 12 months into the study. A separate two-year study showed that as little as 1.5 mg/kg/day could induce an increase in percent days spent in estrus. This increase was marginal and was not statistically-significant though. Furthermore, the increase in days in estrus seen at this dose was apparently not of the magnitude to increase a female rats risk of mammary cancer as there was not an increase in mammary tumor incidence or decrease in mammary tumor onset at this dose in this study. The next highest dose in the same study (3.1 mg/kg/day) also resulted in an increase in days in estrus. The increase in days estrus seen in this study was slightly greater than the increase seen at 1.5 mg/kg/day, but was still not statistically-significant. It was apparently enough to cause an increase an animals risk for mammary cancer as mammary cancer incidences at this dose were increased about two-fold over concurrent control values.

9.4.2 Atrazine Exposure Results in an Earlier Onset of Increased Serum Estradiol Levels

Atrazine exposure of only three months resulted in an increase in serum estradiol levels in SD females. Levels equivalent to those seen at three months were not seen in control animals in this study until nine months. Because there was no intermediate timepoint between three and nine months in this study it is difficult to determine just when the control animals achieved serum estradiol levels equivalent to those seen in the dosed animals at three months. Clearly, though, the dosed animals had higher serum estradiol at three months than the controls did -- indicating that this parameter of reproductive aging was achieved earlier in the dosed rat than in the control rat.

9.4.3 Atrazine Exposure Results in an Earlier Onset of Attenuated LH Surges

Atrazine exposure at high levels (40 and 200 mg/kg/day) was able to attenuate the preovulatory LH surge after only one month of exposure. Exposure to atrazine at lower levels (29.4 mg/kg/day) significantly weakened the LH surge after six months exposure. The lowest dose of atrazine that was able to induce a weakening of the LH surge was 3.65 mg/kg/day, but this weakening was not statistically-significant. It is not clear exactly when a normally aging animal would be expected to experience an weakened LH surge, but the studies described here showed that the control animals did not experience a weakened LH surge when the concurrently run dosed animals did.

9.4.4 Atrazine Exposure Results in an Earlier Tumor Onset

Tumor onset times were consistently decreased following atrazine exposure. The decrease in tumor onset times implies that the process of tumor formation is occurring at an earlier chronological age due to atrazine exposure. Tumor incidence rate were not always increased following atrazine exposure- they were not increased in Thakur, 1992a and only the trend for fibroadenomas was increased in Thakur, 1991a. Tumor onset times were decreased in every study in which they were examined - Thakur, 1991a and 1992a; and Morseth, 1998.

9.5 Pituitary Adenomas

9.5.1 Onset of Pituitary Alterations Following Atrazine Exposure

Table 5-5 provides evidence that the latency period of pituitary adenomas is decreased following atrazine exposure. Pituitary adenomas are known to be age-related in the rodent. The time to onset of other age-related pituitary alterations may also be decreased in atrazine exposed SD females. As described above under section 9.1, pituitary weights and incidences of pituitary hyperplasia are also increased in the untreated female SD rat with age. There is evidence that an early onset of increased pituitary weights and an early onset of pituitary hyperplasia may also be occurring in response to atrazine exposure. Appendix Tables 21 and 22 display absolute and relative (to body weight) pituitary weights from Thakur, 1991a. Appendix Table 23 displays absolute and relative (to body) pituitary weight from SD females exposed to atrazine for six months in Morseth, 1996b.

Three months of exposure did not result in an increase in either absolute or relative- to-body pituitary weights in the dose groups compared to controls. By nine months of exposure there was a clear dose-related increase in both absolute and relative pituitary weights. The effect was still evident at 12 months, but only at the high dose and the effect was less severe at the high dose at 12 months compared to nine months. Pituitary weights at 15, 18 and 24 months are comparable in dose groups compared to controls. The incidence of pituitary focal hyperplasia (as recorded in the histopathology records of this study) was marginally increased in dose groups compared to the control at nine months. There was only one incidence of this histology finding in the control group (it was assigned the grade "slight" by the examining pathologist) while there were two incidences at 4.23 mg/kg/day (both "slight") and two incidences at 26.23 mg/kg/day (one "minimal" and one "slight"). The early onset of increased pituitary weights following atrazine exposure seen in Thakur, 1991a is confirmed in Morseth, 1996b. Both absolute and relative pituitary weights increases are >20% at 29.4 mg/kg/day after six months of exposure.

9.5.2 Role of Early Onset of Pituitary Alterations in Mammary Carcinogenesis

The pituitary alterations - adenomas, hyperplasia and increased pituitary weight - all result in increased serum prolactin levels (Baird *et al.,* 1990; McComb *et al.,* 1986; van Putten *et al.,* 1988). The association between increased prolactin exposure and mammary tumors has been well-described (Meites, 1972; Meites, 1981; Russo *et al.*, 1990). Likewise, an association between pituitary alterations of the types mentioned above and mammary tumors had been well-described (Blankenstein *et al.,* 1984; McConnell, 1989a; Goya *et al.,* 1990). The studies described in this document also provide evidence of an association between these pituitary alterations and mammary tumors. Appendix Table 24 shows that majority of SD females with mammary tumors also had pituitary adenomas. Females without mammary tumors also had high incidences of pituitary adenomas, but the incidences were generally lower than for animals with mammary tumors. Appendix Tables 25a and b show that pituitary weights in females with mammary tumors were higher than pituitary weights in females without mammary tumors. The mean absolute pituitary weight in animals with mammary tumors in Morseth, 1998 was 46% greater than the mean pituitary weight in animals that did not have mammary tumors. The difference was even more apparent in the Thakur, 1992a study where the mean absolute pituitary weight in females with mammary tumors was 94% greater than in those without mammary tumors.

9.5.3 Pathogenesis of Pituitary Alterations

As has been previously discussed, estrogen is mitogenic to the pituitary lactotrophs of the rodent. It is therefore biologically plausible that the same increase in anovulation and accompanying prolonged exposure to serum estrogens that is believed to contribute to mammary carcinogenesis following atrazine exposure would also contribute to pituitary hyperplasia and neoplasia.

If an increase in serum estrogens leads to the afore-mentioned pituitary alterations then it would be reasonable to expect that increases in serum estrogen would precede the pituitary alterations. Indeed, increases in serum estradiol are seen as early as three months following the initiation of atrazine exposure (Appendix Table 15). Dose-related increases in acinar development (a histomorphologic alteration highly dependent on estrogen) are found following three months of exposure while dose-related increases of galactoceles, secretory activity and other histomorphologic alterations indicating prolactin exposure are not seen until nine months of exposure (Appendix Tables 16 and 17).

If estrogens derived from unovulated follicles contribute to pituitary alterations then OVX animals would be expected to have lower incidences of these pituitary alterations. The OVX animals in Morseth, 1998 did have lower incidences of pituitary adenomas, but not as much less as might be expected. As previously noted, ovariectomy was able to reduce mammary tumor incidences from about 50% having some sort of mammary tumor to zero percent having any sort of mammary tumor. The effect of ovariectomy on the pituitary adenoma rate was much less pronounced. Ovariectomy dropped the pituitary adenoma rate from about 70% at terminal sacrifice for the intact animals to about 50% at terminal sacrifice in the OVX animals. Interestingly, though ovariectomy had only mild impact on pituitary tumor incidences, ovariectomy was able to dramatically reduce pituitary weights at the end of the study. Absolute pituitary weights at terminal sacrifice in the OVX animals were only about a quarter the value of the intact animals. Relative pituitary weights were only 35% the weight in OVX compared to intact animals. Appendix Table 26 displays these data. The dramatic decrease in pituitary weight in conjunction with only a mild decrease in pituitary adenoma incidence may be explained by an earlier onset of the pituitary tumors in the intact animals compared to the OVX. The data in Appendix Table 26 provides

evidence of an early onset of pituitary adenomas in intact animals versus OVX when it shows a 6% incidence of pituitary adenomas in the interim sacrifice OVX animals compared to a 17% incidence in interim sacrifice intact animals. The data in Appendix Table 26 also shows that OVX animals had a much reduced incidence of "enlarged" pituitaries compared to intact animals. The decreased incidence of enlarged pituitaries and the reduced weight of the pituitaries may indicate that, though many OVX animals still got pituitary tumors, these tumors occurred later in life and thus, by the time the animals were sacrificed, had not had as much time to grow and were thus of a smaller size.

A serial sacrifice study comparing OVX and intact animals would be useful in determining if pituitary tumor onset is delayed in OVX animals versus intact animals. While HED is not interested in pituitary tumor onset in OVX animals *per se*; the fact that pituitary tumors are not more dramatically decreased in OVX animals raises some doubts about a mode of action for atrazine-mediated pituitary tumors that depends on prolonged exposure to follicular-derived estrogen. Were the same mode of action to apply to pituitary tumors that applies to mammary tumors then one would expect pituitary tumors to behave like mammary tumors when the ovaries are removed. That is, one would expect pituitary tumor rates to drop to zero, or close to zero, following OVX.

9.5.4 Summary and Conclusion for Pituitary Alterations

There is ample evidence in the open literature that exposure to follicular-derived estrogen in CE rats leads to an increased incidence of prolactin-secreting pituitary adenomas and increased pituitary weight or focal hyperplasia. Being that atrazine exposure seems to result in an early onset of constant estrus and increased estradiol exposure, one would expect that these pituitary alterations would also show an early onset following atrazine exposure. Pituitary weight data from Thakur, 1991a and Morseth, 1996 both show an early onset of increased pituitary weight following atrazine exposure. Pituitary adenoma data from Thakur, 1991a shows that there is an early onset of pituitary adenomas following atrazine exposure.

Further research is desirable into why ovariectomy does not reduce pituitary tumor incidence to the same extent as it does mammary tumors incidence despite there apparently having the mode of action.